Chitinase-Like Proteins Are Autoantigens in a Model of Inflammation-Promoted Incipient Neoplasia

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Abstract

An important role for B cells and immunoglobulin deposition in the inflammatory tumor cell environment has been recognized in several cancers, and this is recapitulated in our murine model of inflammation-associated carcinogenesis: transgenic mice expressing the Epstein-Barr virus oncogene LMP1 in epithelia. Similarly in several autoimmune disorders, immunoglobulin deposition represents a key underlying event in the disease process. However, the autoantigens in most cases are not known. In other studies, overexpression of the enzymatically inactive mammalian chitinase-like proteins (CLPs) has been observed in a number of autoimmune disorders and numerous cancers, with expression correlated with poor prognosis, although the function of these proteins is largely unknown. We have now linked these observations demonstrating that overexpression of the CLPs renders them the targets for autoantigenicity during carcinogenic progression. We show that the CLPs, Chi3L1, Chi3L3/YM1, and Chi3L4/YM2, are abundantly overexpressed in the transgenic epidermis at an early, preneoplastic stage and secreted into the serum. Immunoglobulin G reactive to the CLPs is detected in the serum and deposited in the hyperplastic tissue, which goes on to become inflamed and progressively dysplastic. The CLPs are also upregulated in chemical carcinogen-promoted lesions in both transgenic and wild-type mice. Expression of the related, active chitinases, Chit1 and AMCase, increases following infiltration of inflammatory cells. In this model, the 3 CLPs are autoantigens for the tissue-deposited immunoglobulin, which we propose plays a causative role in promoting the inflammation-associated carcinogenesis. This may reflect their normal, benign function to promote tissue remodeling and to amplify immune responses. Their induction during carcinogenesis and consequent autoantigenicity provides a missing link between the oncogenic event and subsequent inflammation. This study identifies the CLPs as important and novel therapeutic targets to limit inflammation in cancer and potentially also autoimmune disorders.

Keywords

chitinase-like protein, carcinoma, inflammation, LMP1, EBV

Introduction

Inflammation is a natural immune response to combat infection and promote wound repair, which should abate when the infection or injury resolves. It is characterized by the infiltration of immune cells into tissues, where they release and respond to factors in a dynamic state. In some conditions, such as allergic asthma, inflammatory bowel disease (IBD), and rheumatoid arthritis (RA), a state of chronic inflammation exists. It is unknown what causes or perpetuates this state, but the tissue is constantly under the bombardment of immune factors, which, instead of promoting repair, underlie the symptoms. Chronic inflammation has also been linked to increased cancer risk, the paradigm for which is the inflammation caused by *Helicobacter pylori* (*H. pylori*) and its association with gastric cancer.^{1,2} That carcinoma cells influence cells within the microenvironment to stimulate inflammation, which then supports tumor growth and metastasis, has been elegantly demonstrated.^{3,4} However, the factors that initiate the inflammatory process are unknown.

Recently, chitinase family proteins have come under increasing scrutiny due to their overexpression in tissues

that are chronically inflamed as well as in several cancer cells and the plasma of these patients. Chitinases catalyze the hydrolysis of chitin, a polymer of N-acetylglucosamine, while the chitinase-like proteins (CLPs) are structurally related but thought to lack enzymatic activity due to substitutions of catalytic residues in the active site. The 2 mammalian active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase), are common to mice and man, and indeed, *Xenopus* has 2 active chitinases (reflecting an ancient duplication of the gene). The CLPs

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appear to have evolved from the active chitinases by more recent gene duplication events followed by loss of function mutations, giving 3 genes in humans and 6 expressed genes in mice.⁵ While the CLPs lack enzymatic activity, they retain the carbohydrate binding domains and are hence also termed chi-lectins. The CLPs include oviductin (Ovgp1) and chitinase 3-like 1 (CHI3L1, also known as YKL-40 or CGP-39) found in both mice and man, CHI3L2 in man, and Chi3L3 and Chi3L4 in rodents (alternatively termed YM1 and YM2, which will be used in this report), as well as the relatively uncharacterized murine genes, basic YM (bYM) and brain chitinase-like protein 2 (Bclp2).⁵⁻⁷ Of 2 further YM genes identified in the mouse, YM3 (Chi3L5) is not known to be expressed, and the incomplete YM4 is probably a pseudogene.⁵ Murine YM1 and YM2 share >95% amino acid sequence similarity but show differing tissue expression patterns, with YM1 normally expressed predominantly in the lung, spleen, and bone marrow, while YM2 is expressed in the murine forestomach and weakly in the thymus and kidney.^{8,9}

Active chitinases are induced in diseases involving chitin-containing organisms but have also been implicated in several disorders in which a role for such pathogens has not been demonstrated. AMCase and chitotriosidase were detected at elevated levels in the epithelial and inflammatory cells in nasal polyps, compared to surrounding tissue,¹⁰ and the latter in the serum of multiple sclerosis and juvenile arthritis patients^{11,12} as well as certain cancers.¹³ As well as AMCase and CHIT1, there is increasing evidence that several CLPs, notably CHI3L1, have an association with chronic inflammation and tissue remodeling, with overexpression observed in autoimmune diseases, allergy, wound healing, infection, and several cancers and indeed can be prognostic indicators for some cancers.¹⁴ It is becoming apparent that these are more than just correlated phenomena but that the proteins contribute to the various pathologies.

Chitin is the second most abundant natural polysaccharide (after cellulose), produced by fungi, crustaceans, helminths, insects, and many others, to more than one billion tons per year. It is not produced by mammals; however, degradation of this stable and insoluble polymer is important for immune defense against chitin-containing organisms. Infection of mice with a helminth was found to lead to inflammation and induction of AMCase as well as YM2.¹⁵ Indeed, treatment of mice with chitin alone induces the infiltration of innate immune cells implicated in allergic reactions. However, in mice overexpressing AMCase, the chitin-induced inflammation was attenuated, suggesting that the role of AMCase is a negative feedback one since it effectively removes the chitin stimulus.¹⁶ However, what of the CLPs, which may bind to chitin or other polysaccharides but not degrade them? These could therefore be mediators of inflammation. Indeed, induction of CLPs has now

been reported in several cases of inflammation in mice and man, not necessarily involving chitin-containing organisms as the stimulus. Expression of human CHI3L1 or CHI3L2 was found to be induced in patients with RA.¹⁷ CHI3L1 is induced in colonic epithelial cells during active but not quiescent IBD.¹⁸ Elevated serum levels of CHI3L1 are found in patients with asthma and correlate with severity,¹⁹ and elevated CHI3L1 in cerebrospinal fluid is associated with conversion to multiple sclerosis.²⁰ Expression of YM1 in the mouse increases dramatically during parasitic infections,²¹⁻²⁴ and YM2 was found to be upregulated in a mouse model of allergic pulmonary inflammation.^{25,26} In addition, YM1 and YM2 were shown to be induced in the skin of a mouse mutant with chronic proliferative dermatitis (the cpdm mouse) as well as in mice with induced contact hypersensitivity. In these mice macrophages, dendritic cells and mast cells were identified as the cellular sources.²⁷

In addition to autoimmune and inflammatory disorders, high expression levels of CHI3L1 in the tumor tissue and/or levels of secreted protein in the serum have been observed with several cancer types, including breast, ovarian, gastric, prostate, and colon cancer and Hodgkin's lymphoma among others. Furthermore, high levels of CHI3L1 are correlated with poor prognosis,^{14,28} and the protein has been found to be a contributory factor in tumor-associated angiogenesis.²⁹ As such, induced levels of a CLP reflect an inflammatory or cancerous condition and may further demonstrate the sequential link between these states.

Following a differential proteomic analysis using our L2LMP1^{CAO} transgenic mouse model of multistage epithelial carcinogenesis, we found substantial overexpression of YM2 in the transgenic pathological tissue.^{30,31} Latent membrane protein 1 (LMP1), an Epstein-Barr virus (EBV) oncoprotein, induces NFκB, JNK, and STAT signaling pathways, which are activated in the skin of the L2LMP1^{CAO} mice, as are the p38 and ERK mitogen-activated protein kinases.³² NFκB is a prominent mediator of inflammation, regulating the expression of several proinflammatory cytokines.^{33,34} The skin of the L2LMP1^{CAO} mice, most notably of the hairless regions, particularly the ears, shows evidence of hyperplasia soon after birth. The condition progresses with time, and by 6 months of age, all mice show extensive dysplastic dermatitis of the ears, with inflammation, ulceration, and necrosis. This can lead to the development of keratoacanthomas, papillomas, and occasionally carcinoma. We have shown that the transgenic tissue becomes infiltrated with T cells, mast cells, and neutrophils, and immunoglobulin G (IgG) is deposited.³⁵ The affected tissues of these mice show upregulation of several chemokines and cytokines as the pathology progresses, including CD30 and its ligand (CD153), CXCL13, CXCL10, L-selectin, IL-3, IL-1β, TGFβ1, and macrophage inflammatory proteins among others.³⁵ The importance of the lymphocytic infiltrate was

demonstrated by genetic elimination of mature B cells and T cells, which halts progression of the pathology, limiting it to an early benign stage.

In order to explore the role of chitinases and CLPs in inflammatory conditions and carcinogenesis, we have profiled the expression patterns of these proteins as the pathology progresses in this oncogene-driven mouse model. We have identified a link between IgG deposition in the tissue, an early feature in the inflammatory process, and abundant overexpression of 3 CLPs; these proteins become prominent autoantigens.

Results

YM1 and YM2 are hugely induced in the transgenic epidermis. We have previously described the phenotype of the L2LMP1^{CAO} mice, which presents predominantly in the skin of hairless body regions, particularly the ears.³⁰ To facilitate study, we have categorized the preneoplastic phenotype into 5 recognizable and predictable stages, from stage 1 (St1) in neonates and up to approximately 1 month old, showing mild hyperplasia with increased vascularization, to stage 5 (St5), from about 6 months old, displaying severe hyperplasia, hyperkeratosis, parakeratosis, erosive or ulcerative dermatitis, and necrosis with fibrovascular hyperplasia of the underlying dermis, which can lead to keratoacanthoma and occasional carcinoma in older mice.³⁰ In a differential proteomic analysis of the preneoplastic tissues, the protein found to be upregulated to the highest degree in the transgenic samples was identified by mass spectrometry as YM2 (Fig. 1).³¹ In order to confirm and assess the degree of overexpression in the transgenic samples, replicates from 2 independent transgenic lines, L2LMP1^{CAO}.117 and L2LMP1^{CAO}.105B were examined by western blotting and compared to transgene-negative sibling control (NSC) replicates. YM protein was found to be massively upregulated in all stages in the transgenic samples of both lines (Fig. 1C and 1D), thereby demonstrating that this observation is not unique to one line. YM was upregulated in the transgenic tissues at an early age, seen in 4-week-old St1 samples (Suppl. Fig. S1). Two prominent bands by western blotting (which show increasing relative proportion of the upper band with increasing mouse age and sample stage) were observed at approximately 39 and 44 kD (Fig. 1). The YM protein levels detected in the transgenic tissues were very high, being readily detectable by western blotting, even when loading relatively small amounts of total protein (as little as 1 µg [not shown]) on the gels. Very low levels of the 44-kD band only were detected in control samples when using anti-YM1 antibody (Fig. 1C) but not using anti-YM (DW) antibody (Fig. 1D); as such, the fold increase in the transgenic samples was difficult to determine.

The antibodies used recognize both of the very closely related YM1 and YM2 proteins. In order to assess which or if both genes are overexpressed, RT-PCR using discriminatory primers was conducted using RNA from the affected tissue and transgene-negative controls. YM2-specific cDNA could be amplified from the transgenic ear tissue but was not detected in NSC (Fig. 1E), clearly demonstrating upregulation of YM2 RNA. YM1-specific cDNA could not be detected from transgenic or control tissue samples, despite amplification from the genomic DNA control (Fig. 1E). To explore this further, YM was immunoprecipitated from 1 mg of St2, St5, and NSC ear tissue protein extracts and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with colloidal Coomassie blue. The clear 39-kD and 44-kD bands present only in the transgenic tissue samples (not in controls) were isolated and subjected to MALDI-ToF mass spectrometry. The 44-kD band was identified as YM1 and the 39-kD band as YM2 by peptide stretches unique to each protein (Suppl. Fig. S2). This indicates that both proteins are strongly upregulated in the transgenic tissue, showing an increasing ratio of YM1 (44 kD) to YM2 (39 kD) as the mice age (Fig. 1 and Suppl. Figs. S1 and S2).

In order to examine YM expression through carcinogenic progression, L2LMP1^{CAO}.117 transgenic and NSC mice were treated with a classic regime of topical chemical carcinogens to induce dorsal skin papillomas and carcinomas.³⁶ Lesions induced in this manner in wild-type mice typically harbor an activated *H-ras* allele (among other oncogenic changes through progression). In the LMP1 transgenic lesions, YM1 and YM2 expression was further induced (Fig. 1F) to even higher levels than detected in the preneoplastic staged samples (Suppl. Fig. S1). Interestingly, in transgene-negative controls, YM1 and YM2 were also induced in the papillomas and carcinomas (albeit to a lesser extent than the transgenic samples) and showed different relative proportions, with the 39-kD/YM2 band stronger in papillomas (Fig. 1F). This demonstrates that induction of YM proteins through carcinogenesis is not unique to the LMP1 transgenic model.

In order to ascertain the cellular location of the YM protein within the tissue, transgenic preneoplastic ear samples and controls were examined by immunohistochemistry. YM protein was detected in the transgenic epidermis, extending in regions from the basal cells through the suprabasal layers to the cornified layers, while none was detected in NSC samples (Fig. 2A-D). In a mouse model of allergic pulmonary inflammation, YM2 expression was found to be upregulated in an interleukin-4 (IL-4)-, IL-13-, and CD4⁺T cell-dependent fashion.^{25,26} In order to investigate if YM expression occurs as a consequence of the inflammation and is similarly dependent on the T-cell infiltrate in our L2LMP1^{CAO} transgenic model, expression was examined in

Figure 1. Upregulation of YM proteins. Following a proteomic comparison by 2-D DiGE between LMP1^{CAO}.117 transgenic ear samples at stage 2 (St2) and stage 5 (St5) with NSC (C2 and C5), the protein YM2 was identified as the most highly upregulated protein in the transgenic extracts.³¹ (A) Two-dimensional (upper panels) and 3-dimensional (lower panels) fluorescence representations of the differentially expressed spots (ringed in fuchsia and subsequently identified as YM2 by mass spectrometry) are shown. (B) Graphical representations of the fluorescence intensity of the selected spots on biological replicate analytical gels using dyes Cy3 or Cy5, with standardized log abundance on the y axis versus sample type on the x axis. The solid black line represents the gel from which the 2-D and 3-D images were taken. The broken lines represent the other analytical gels, and the blue line represents the average of all analytical gels. The fold regulation and significance of the difference (Student t test) are shown in the white box (inset). (C and D) Protein was extracted from transgenic ears (St2 and St5) of the lines L2LMP1^{CAO} 117 and 105B along with negative sibling controls (NSC: C2 and C5). Three biological replicates (50 μ g [C], 40 μ g [D]) were western blotted (10% gels) and probed with anti-YM antisera (commercial antibody used [C], DW antibody used in [D]; the latter does not detect the faint band at 44 kD seen in controls), with anti-GAPDH (as indicated) used as a loading control. Molecular weight markers are shown (kD). (E) RNA from phenotype-staged line 117 transgenic ear tissue (St2 or St5) and NSC (C2 or C5) was used for RT-PCR, performed with (+) or without (-) reverse transcriptase, using YM1- (upper panel), YM2- (middle panel), or GAPDH (lower panel)-specific primers. YM1 genomic DNA product = 2201 bp; expected cDNA product = 624 bp; YM2 cDNA product = 429 bp; GAPDH cDNA product ~500 bp. (One St5 sample was of lower quality or quantity as judged by GAPDH amplification; nevertheless, YM2 was amplified from this sample.) (F) Protein was extracted from chemical carcinogen-induced papillomas (pap) and carcinomas (carc) from transgenic mice of line 117 and NSC. Two or 3 biological replicates (40 μ g) were western blotted (10% gel) and probed with anti-YM antisera (DW) together with anti- β -tubulin antisera (loading control), detected by different wavelength infrared dye-conjugated secondary antibodies (composite image shown). The 44-kD YM band was identified by mass spectrometry as YM1 and the 39-kD YM band as YM2.

L2LMP1^{CAO} mice in a recombinate activating gene 1 (RAG1)-null background (lacking mature B cells, T cells, and NKT cells). In this background, the LMP1-induced ear phenotype is limited to St1 or St2, and no infiltration of mast cells or neutrophils is observed, while RAG1 heterozygotes show an identical LMP1-induced phenotype as in a wild-type background.³⁵ In the RAG1-null background, induced YM was still detected in the L2LMP1^{CAO}

transgenic ear tissue (Fig. 2E-H). By western blotting, the relative levels of the 44-kD/YM1 and 39-kD/YM2 bands were equivalent between the L2LMP1^{CAO} St2 samples in a wild-type background and the St2 L2LMP1^{CAO} samples in the RAG1-null background (Fig. 2). This demonstrates that both YM proteins are expressed in the absence of the inflammatory cell infiltrate observed in a wild-type background.

Figure 2. Expression of YM is induced in the transgenic epidermis. (A-D) Detection of YM protein in 2-month-old, formalin-fixed, L2LMP1^{CAO} transgenic mice stage 2 (St2: B and D) and NSC (C2: A and C) ear tissue sections, from original magnification at 100x (A and B) and 400x (C and D). The epidermal basement membrane is indicated by a black dotted line (C and D). (E-H) Detection of YM protein in ear sections from 6-month-old transgenic mice in either a RAG1-null (E and G) or RAG1-heterozygote (F and H) background, with original magnification at 100x (E and F) and with expansion to 200x of the boxed areas (G and H). (I) Protein was extracted from ear samples from St2 transgenic L2LMP1^{CAO} transgenic mice in a wild-type background (aged 2.5 months old) and age-matched controls (WT, C2) and from St2 transgenic L2LMP1^{CAO} mice in a RAG1-null (-/-) background (aged 6 months old) and age-matched RAG1-null controls (RAG1^{-/-} (C2). In the RAG1-null background, the transgenic skin phenotype (noted particularly in the ears) is slower to develop than in a wild-type background and does not progress beyond St2, hence the older age of the RAG1-null mice used for the assay.³⁵ Two biological replicates of each (50 µg) were separated by 10% SDS-PAGE and western blotted. The blots were probed with anti-YM antisera (DW) (upper panel) and anti-Chi3L1 antibody (middle panel). Anti-GAPDH probing was used as a loading control (lower panel).

Figure 3. Upregulation of Chi3L1. Protein was extracted from (A) transgenic (St2 and St5) and control (NSC: C2 and C5) ears and (B) chemical carcinogen-induced papillomas (pap) and carcinomas (carc) from mice of the transgenic line L2LMP1^{CAO,117}. Three biological replicates of each (50 µg) were separated by 10% SDS-PAGE and western blotted. The blots were probed with rat anti-Chi3L1 antibody (migrates at 39 kD). GAPDH was used as a loading control (lower panels). (C) Formalin-fixed ear tissue sections from L2LMP1^{CAO,117} transgenic (St4) and NSC (C4) were immunostained with anti-Chi3L1 antibody, with original magnifications of 100x (left) and 400x (right).

some samples (Fig. 3) and may reflect a closely sized doublet in several of the samples; however, in the control nontumor samples, the predominant band was the lower one of the doublet (Fig. 3). The detection of 2 bands by western blotting may reflect posttranslational modifications of Chi3L1 (such as phosphorylation) or translation from differentially spliced transcripts (the CLPs generally express multiple splice variants). Expression of oviductin (120 kD), another CLP that is common to mice and man, could not be detected, although a 55-kD unidentified protein detected with the oviductin antibody was upregulated in St5 samples (not shown).

The active chitinases *Chit1* and *AMCase* are induced in the inflamed tissue. The 2 active chitinases, Chit1 and AMCase, were also examined. Human CHIT1 is synthesized as a 50-kD secreted protein, the predominant form found in the blood stream, although 5 differentially spliced coding transcripts have been identified in humans and 4 in the mouse. The 50-kD product is processed to a 39-kD form lacking the chitin binding domain, detected as a doublet that accumulates in lysosomes.³⁷ In the murine ear tissue, we observed a 50-kD protein detected with the antichitotriosidase antibody, which is observed at slightly higher levels in St2 tissue compared to controls, but with greater induction in St5 samples compared to controls (approximately 10-fold) (Fig. 4A). In addition, a doublet of approximately 30 kD was detected, which was expressed at significantly higher levels in the transgenic St5 tissue compared to age-matched controls (C5). This intense doublet in the St5 samples was poorly detected in samples that were not reduced (Fig. 4B), indicating that disulphide bonds are integral to the structure of this protein. It is not clear if this doublet at 30 kD is processed murine chitotriosidase, or the product of a differentially spliced *Chit1* transcript, or if this reflects affinity of the antibody for a related protein that is also upregulated in the St5 transgenic tissue. Compared to the level of expression of the 50-kD Chit1 in the St5 tissue, little Chit1 is detected in chemically induced papillomas and carcinomas, which show no difference between transgenic-positive and -negative control samples (Fig. 4B).

AMCase migrates at 50 kD by SDS-PAGE to avoid obscuring detection by the immunoglobulins (the heavy-chain IgH migrates at 55 kD), which are present in high levels in St5 tissue; these samples were assayed without reduction, with the nonreduced immunoglobulins migrating slowly at the gel top. AMCase was not detected in control tissue samples at all; however, clear expression was observed in the transgenic St5 extracts (Fig. 4C). Confirmation that this was not IgH crossreactivity was obtained by reprobing the blot with secondary antibody alone, which showed no reactivity to this band. No AMCase was detected in papilloma and carcinoma samples (not shown). Thus, the active chitinases Chit1 and AMCase are both upregulated in the late

Figure 5. Secretion of YM, Chi3L1, and Chit1. **(A)** Serum samples (5 μ L/track) from transgenic line L2LMP1^{CAO} 117 mice with St5 ear phenotype and NSC mice (C5) were separated by 10% SDS-PAGE and western blotted. The membranes were probed with anti-YM (DW), anti-Chi3L1, anti-Chit1, or anti-AMCase antibodies as indicated. Samples for Chit1 and AMCase detection were not reduced with 2-mercaptoethanol. Reduced immunoglobulin heavy chain (IgH) is indicated in the upper panel. As controls, 10 μ g tissue extracts from transgenic ear St5 and NSC (C5) were also loaded. **(B)** Protein was extracted from 3 LMP1 transgene-positive cell lines (+), 105B.113, 117.990, 105B.92, and 3 transgene-negative cell lines (-), 105B.110, 117.30, and 105B.60, derived from carcinomas from mice of the lines L2LMP1^{CAO} 117 and 105B as the notation indicates, using 20 μ g protein per track (50 μ g for 105B.60 and 105B.92 cell lines). Also loaded were 30 μ L of conditioned medium from the respective confluent cultures and 20 μ g tissue extracts from transgenic ear tissue St5 and NSC (C5). Proteins were separated by 10% SDS-PAGE and western blotted. The membrane was probed with anti-YM antibody (upper panel) and anti-Chi3L1 antibody (lower panel). Repeat blots show no CLP expression in cell line 105B.60, partly obscured here by YM2 levels seen in the adjacent track. Molecular weight markers are shown (kD).

preneoplastic stages (St5) when the tissue is heavily inflamed, but neither is induced in papillomas or carcinomas.

The CLPs and Chit1 are secreted. Elevated CHI3L1 and, in some cases, CHIT1 levels have been found in the serum of patients with inflammatory disorders and several cancers. In these mice, YM (predominantly the 44-kD band identified as YM1) and Chi3L1 were detected in the serum of the L2LMP1^{CAO} mice but not in NSC mice (Fig. 5A and Suppl. Fig. S3). Intriguingly, Chi3L1 was also detected in the serum of RAG1-null mice, both LMP1 transgene negative and positive (Suppl. Fig. S3); the reason why this might differ from wild-type mice is currently not understood. The 50-kD secreted enzymatically active Chit1 was also detected at higher levels in the transgenic serum compared to controls; however, AMCase was not detected in the serum of transgenic mice or controls (Fig. 5A).

Expression of the CLPs was also examined in cell lines established from the chemically induced carcinomas. Three transgene-positive cell lines derived from mice of lines 117 and 105B were examined, one of which (105B.92) was

found to express high levels of the 39-kD YM protein (identified as YM2 above) and secrete large amounts into the medium (Fig. 5B). Secretion of YM2 was also observed with the transgene-positive cell line 105B.113. Transgene-positive cell line 117.990 instead secreted Chi3L1 with little or no YM (Fig. 5B). A number of other transgene-positive cell lines were examined and found not to express the 3 CLPs (not shown). Although chemically induced carcinomas arising in transgene-negative mice were found to express YM1, YM2, and Chi3L1 (Figs. 1 and 3), several cell lines derived from these showed no expression or secretion of these (3 representative cell lines shown in Fig. 5B).

Chi3L1 and YM proteins are autoantigens. We have shown that the transgenic dermis develops a heavy deposition of immunoglobulin, and IgG is highly abundant by St5.³⁵ Moreover, deficiency of mature B cells and T cells limits the pathology to an early, benign stage, indicating that lymphocytes or their products are critical to the progression of the pathology.³⁵ What drives the immunoglobulin deposition, in other words, what are the antigens, is a critical question in

Figure 6. Transgenic serum contains antibodies directed toward YM2 and Chi3L1. **A** and **B**: Protein extracts (50 µg) from transgenic line L2LMP1^{CAO}.117 (St2 and St5) and NSC (C2 and C5) ear samples were separated by 10% SDS-PAGE and western blotted. The membrane was first probed with serum pooled from four NSC (C5) mice (**A**), then stripped and reprobed with serum pooled from four transgenic mice with St5 ear phenotype (**B**). Both 5 second exposure time. Molecular weight markers are indicated (kD). **C-F**: Protein extracts (200 µg) in NET-N buffer from ears of line L2LMP1^{CAO}.117 transgenic mice (St2) and control (NSC: C2) were immunoprecipitated with 4 µL of anti-YM antibody (**C** and **E**) or 2 µL of anti-Chi3L1 antibody (**D** and **F**). The immunoprecipitated proteins were separated by 10% SDS-PAGE using replicate gels, along with 50 µg RIPA ear tissue extracts of transgenic St2 or St3 and NSC C2 or C3 and western blotted. **C** and **D**: The membranes were probed with serum pooled from four control mice (NSC serum, upper panels), then stripped and reprobed with serum pooled from four transgenic mice with St5 ear phenotype (lower panels). **E** and **F**: Replicate membranes were sequentially probed with anti-Chi3L1 antibody then anti-YM antibody as indicated. Molecular weight markers are indicated (kD). Arrows indicate the Chi3L1 or YM bands as detected by specific antisera.

determining the early causal events in the pathology of this model. Human CHI3L1 and CHI3L2 have been found to be rheumatoid arthritis auto-antigens in some patients.³⁸ In order to investigate if the overexpressed chitinases and CLPs detected in this model are autoimmune targets in the affected mice, western-blotted tissue extracts were probed with either transgenic or control serum and detected with anti-murine-IgG secondary (Fig. 6). The proteins within the tissue extracts detected by the transgenic serum showed a similar western-banding pattern to those detected with control serum (Fig. 6A and 6B), however they were observed at a consistently greater intensity using the transgenic serum. Most of the detected bands were observed at equal levels in the transgenic and matched control tissue extracts, however (as well as IgH at 50 kD) a notable band at approximately 39 kD was more abundant in the transgenic tissues compared to the controls and was readily detected by the transgenic serum (and faintly by NSC serum). In order to determine if this antiserum detected protein is a CLP, extracts from St2 and NSC tissues were immunoprecipitated using anti-YM or anti-Chi3L1 antisera and immunoblotted (Fig. 6C to 6F). Replicate blots were then probed with sera either derived from L2LMP1^{CAO}-positive transgenic animals at St5 or from NSC; these were subsequently stripped and reprobed with antisera to YM or Chi3L1 (which show no crossreactivity to the reciprocal proteins). Following YM immunoprecipitation, a band at 39 kD is evident in the transgenic samples, not present in NSC samples, when probed with transgenic serum, which is absent when probed with sera from NSC (Fig. 6C). This band exactly migrates with the YM2 band subsequently detected with anti-YM specific

antisera. Antisera to Chi3L1 (which migrates marginally faster than YM2) do not detect this YM precipitated protein (Fig. 6E). Similarly, following Chi3L1 immunoprecipitation, a band at 39 kD is evident in the transgenic samples but not detected NSC samples, when probed with transgenic serum and very faintly detected by NSC serum (Fig. 6D). This band exactly comigrates with the band detected with anti-Chi3L1 specific antisera. Antisera to YM do not detect this Chi3L1 precipitated protein (Fig. 6F). This demonstrates that antibodies that specifically recognize YM2 and Chi3L1 are circulating in the transgenic mouse serum. It is also suggestive that low levels of antibodies to Chi3L1 are present in the sera of control mice (also seen in Fig. 6A).

To investigate if the immunoglobulins deposited within the affected tissue also include the autoantibodies detected in serum against the chitinases and CLPs expressed within the tissue, a “self-IP” approach was used. Immune complexes were allowed to form between the immunoglobulins and the proteins present within the transgenic or NSC ear tissue extracts from both lines L2LMP1^{CAO}.117 and L2LMP1^{CAO}.105B. The complexes were immunoprecipitated using protein G, which specifically binds to IgG (and not IgA, IgE, or IgM). The blots of these samples were then probed with antisera to YM, Chi3L1, or Chit1 (Fig. 7). YM bands were detected in the transgenic samples from both lines and not in NSC samples. Direct comparison with a total protein transgenic extract indicates that approximately 1/50 of the YM protein present within the sample was immunoprecipitated by the tissue IgG (although protein G sepharose may have been limiting) (Fig. 7A). Similarly, Chi3L1 was also clearly immunoprecipitated from the transgenic tissue by the IgG present within the tissue extract, but not in NSC samples (Fig. 7B). For Chit1, the samples were not reduced to minimize IgH (at high levels in the St5 samples) masking the Chit1 50-kD band; however, the samples resolve less well as a consequence (Fig. 7C). A band at 50 kD can be seen in the transgenic St5 samples not observed in the controls, comigrating with the 50-kD band detected in complete (non-IP) samples. While some ambiguity remains that this band could still reflect IgH detection, it is suggestive that Chit1 is also an autoantigen within the tissue.

Together, these data demonstrate that the CLPs, YM1, YM2, and Chi3L1, are autoantigens (and possibly also the chitinase Chit1) in this transgenic mouse model and that the antibodies to these proteins are present in the serum of the transgenic mice and are deposited in the pathological tissue. Furthermore, unless the transgenic sera are coincidentally reacting to unknown autoantigens of the same size as YM2 and Chi3L1 (39 kD), induced in the transgenic tissue, YM and Chi3L1 are major targets of the auto-IgG response in the transgenic mice.

Discussion

The ears of the L2LMP1^{CAO} mice (and to a lesser extent, other regions of body skin) develop a pathology of hyperplasia with increased vascularization, progressing to acanthosis with hyperkeratosis, parakeratosis, and erosive or ulcerative dermatitis, leading to the development of keratoacanthoma and papilloma and ultimately carcinoma. Examination of the preneoplastic stages has revealed that the tissue becomes increasingly inflamed with infiltrates of T cells, mast cells, and neutrophils; that IgG is deposited; and that several cytokines and chemokines involved in inflammation are deregulated.³⁵

Extending far back in evolution, active chitinases have had a major role in host defense mechanisms to degrade invading chitin-containing pathogens, while in chitin-containing organisms, the enzymes are essential to remodel their structure.¹⁷ It is intriguing to consider that the mammalian CLPs might also have roles in host defense mechanisms and tissue remodeling, but in the absence of enzymatic activity and the substrate chitin.

The murine chitinase-like YM proteins were found to be abundantly upregulated in the epidermis of this transgenic model, clearly detected in the keratinocyte cytoplasm, and also secreted into the serum. Mass spectrometric analysis of the 2 observed YM antibody reactive bands at 44 kD and 39 kD identified these as Chi3L1/YM1 and Chi3L4/YM2, respectively. Induction was observed in young transgenic mice, before the onset of detectable inflammation (at 1 month old, St1), which suggests that the YM genes are targets of LMP1 signal transduction, either intrinsic to the cell through direct LMP1 activation of NF- κ B, JNK, and STAT pathways or in *trans* through the upregulation by LMP1 of other growth factors such as TGF α and activation of the subsequent pathways.^{30,32} Moreover, YM1, YM2, and Chi3L1 were still overexpressed in the absence of mature B and T-cells (in RAG1-null mice), demonstrating that the induction of these CLPs was not dependent upon T cell-specific cytokines. Following chemical carcinogenesis, YM1 and YM2 were upregulated in L2LMP1^{CAO} transgenic and wild-type mouse lesions, but to a greater extent in the transgenic samples. This overexpression was observed in the papillomas and carcinomas, which were collected between 3 and 11 weeks after the cessation of carcinogen treatment and therefore probably reflects expression induced via an activated cellular oncogene route rather than the direct impact of the chemical TPA, with the action of LMP1 augmenting the upregulation of YM.

Chi3L1, a CLP with homologues in mice and man, was also upregulated from an early age in the transgenic epidermis, again suggesting that this results from LMP1 action in the epithelium and is not solely due to the subsequent inflammation. Chi3L1 was also induced in papilloma and

Figure 7. Transgenic ear tissue contains antibodies directed towards YM1, YM2, and Chi3L1. Protein was extracted from transgenic (tg) L2LMP1^{CAO} 117 and 105B (St5 and St3, respectively) and control (NSC: C5 and C3) tissues into NET-N for self-immunoprecipitation (self-IP) using 200 μ g of protein per sample. Endogenous antibody/antigen complexes were immunoprecipitated and separated by 12.5% SDS-PAGE (10% [C]) and western blotted, along with 50 μ g L2LMP1^{CAO}. 117 St5 and NSC:C5 RIPA ear extract as controls (tissue). (C) Samples were not reduced. The membranes were probed with anti-YM antibody (A), anti-Chi3L1 antibody (B), or anti-Chit1 antibody (C); arrows indicate the specific bands. The immunoglobulin heavy (IgH) and light chains (IgL) are indicated at 55 kD and 25 kD, respectively. Molecular weight markers are indicated (kD).

carcinoma samples, to a similar degree in wild-type and transgenic mice, which could indicate a role in carcinogenesis independent of LMP1 action.

While all 3 CLPs, Chi3L1, YM1, and YM2, were abundantly expressed in the carcinoma tissues, transgenic cell lines developed from such carcinomas showed no or variable expression, and carcinoma cell lines derived from wild-type mice showed no expression. Also, the expression of the CLPs was not coincident in the transgenic cell lines, with 2 expressing and secreting YM2 and not YM1 or Chi3L1 and a third cell line secreting Chi3L1 and not YM1 or YM2. Furthermore, we have observed that expression of YM2 in later passages of the high expressing cell line (105B.92) shows reduced expression of YM2. This suggests that the control of expression of these proteins is not identical, and while LMP1 activity may factor into this, it is complex and likely to depend upon the exact cellular context. These data also demonstrate that while these proteins could be factorial during carcinogenesis, neither protein is essential for survival of the established cell lines in culture, transgenic, or wild type, although this does not address if this is true for the tumors *in vivo*.

The active chitinases Chit1 and AMCase were induced in the transgenic St5 samples compared to controls; however, in contrast to the CLPs, neither was detectably induced in the samples from young mice at St2 when the tissue inflammation is just developing. Expression of Chit1 was observed in papillomas and carcinomas, but at a lower level compared to the inflamed preneoplastic St5

tissue, while AMCase was not detected. Therefore, the induction of these active chitinases in the preneoplastic tissue probably results as a consequence of the inflammatory processes. Basal and prolactin-induced CHIT1 expression in human macrophages was found to be prevented by mitogen-activated kinase p38 and p44/42 (ERK1&2) inhibitors.³⁹ In our mouse model, we have previously found that p38 and ERK1&2 are activated from an early age³²; however, with the detection of Chit1 only in the later inflamed stages, the expression possibly originates from the infiltrating inflammatory cells or is induced by inflammatory factors. This was similar to AMCase, which was only induced in the highly inflamed preneoplastic tissue samples (St4/5). Indeed, treatment with anti-inflammatory drugs in a mouse model of asthma has been shown to suppress the elevated levels of chitinases.⁷

Taken together, these data suggest that the CLPs, Chi3L1, YM1, and YM2, induced early by the oncogenic signaling in the epithelium, could be factorial in initiating the inflammatory process, while the active chitinases, expressed following recruitment of the inflammatory cells, may contribute to the ongoing inflammatory response. In support of the idea that Chi3L1, YM1, and YM2 promote inflammation, these proteins were found to be autoantigens in the transgenic mice. Serum from mice with St5 ear pathology reacted to YM2 and Chi3L1 and antibodies present within the pathological ear tissue reacted to all 3 proteins. No antibody reactivity to YM proteins was detected

in control serum and tissues, however, low-level antibody reactivity to Chi3L1 may be present in the control serum, possibly reflecting the basal expression of this CLP. A prominent feature of the pathology in these mice is the deposition of IgG in the dermis; indeed, in the absence of mature B and T cells, other inflammatory cells including mast cells and neutrophils are not recruited to the tissue, and the pathology is halted at an early benign stage.³⁵ A critical role of B cells in inflammation and carcinogenesis was also observed in another multi-stage epithelial carcinogenesis model in which the driving transgenic oncogenes are the human papilloma virus-16 E6 and E7 proteins.⁴⁰ Immunoglobulin deposition is also characteristic at the inflamed synovial membrane of the joints in rheumatoid arthritis patients. In these patients, type II collagen is an autoantigen along with CHI3L1 and CHI3L2 in some patients,³⁸ and interestingly, CHI3L1 binds to collagen,¹⁴ an association that could be involved in the proposed tissue remodeling activities of the protein. Elevated levels of CHI3L1 have also been found in the cerebrospinal fluid of patients who converted to multiple sclerosis from an isolated syndrome,²⁰ and interestingly, prior infection with EBV is an established risk factor for multiple sclerosis.⁴¹

The loss of tolerance and development of Chi3L1, YM1, and YM2 as autoantigens in this transgenic oncogene-driven model could have been through exposure of a usually concealed epitope, expression of a rare isoform, or an unusual posttranslational modification. Alternatively, it may follow from the aberrant and considerable overexpression through natural antibodies; however, these would be expected to be low affinity IgM and not IgG. Loss or lack of tolerance to these proteins could potentially reflect a normal function and not merely represent abnormality. We suggest a novel concept that these proteins may act as “agents provocateurs,” functioning as natural autoantigens or even adjuvants in their role to promote tissue remodeling and in immune

Figure 8. A working model for the role of chitinases and CLPs in disease. **(A)** Infection by a chitin-containing pathogen, or stimulation by an allergen as in asthma, leading to the production of IL-13 and/or IL-4 by Th2 cells (T) as part of the immune response, in turn stimulates chitinase expression from epithelial cells (E) or inflammatory cells such as macrophages or mast cells (M), as shown for AMCase⁴⁴ and YM2.²⁵ This in turn promotes further inflammation. In the case of a chitin-containing organism, the chitinase contributes to resolution of the infection through its degradation. During tissue remodeling, transient growth or stress signals may serve to induce the CLP, which could also illicit a limited inflammatory response. **(B)** Aberrant oncogene or stress gene activation (in the epithelium in the transgenic mice here) leading to growth and stress signaling can induce expression of CLPs (Chi3L1 and YM in the mice described). Abundant overexpression of the CLPs leads to autoimmune activation and B-cell (B) production of anti-CLP IgG, which is deposited in the CLP-expressing tissue. This in turn leads to inflammatory cell recruitment, including T cells and mast cells or macrophages and induced chitinase expression and possibly further enhanced CLP expression. Chronic inflammation involving further inflammatory cell infiltrates (such as neutrophils [N]) can ensue if the oncogenic stimulus is not negated, which can serve to provide a tumor-supporting environment.

responses to amplify the response, thereby speeding resolution. Chronic CLP overexpression would therefore clearly present a significant problem.

The distinction in the route and timing of induction of the active chitinases versus the CLPs may shed light on their separate functions, and we suggest a working model hypothesizing their distinct roles (Fig. 8). The active chitinases, expressed during the inflammatory process, while augmenting inflammation, also contribute to the resolution of a pathogen infection by degrading the chitin polymer, thereby removing the inflammatory trigger,¹⁶ while the CLPs may serve to enhance the inflammatory signal during this process (Fig. 8A). Similarly, the CLPs may be induced during tissue remodeling or following tissue damage to promote inflammation for repair purposes, which would be resolved when the wound is repaired or the tissue remodeled (Fig. 8A). A B-cell response to CLP could factor in this role. However, CLP induction following aberrant oncogene or stress gene activation may act to establish inflammation

that cannot be resolved without removal or deactivation of the initiating event, which could therefore lead to a chronic state (Fig. 8B).

The initiating trigger(s) in most autoimmune and chronic inflammatory disorders, such as RA, IBD, and multiple sclerosis, is unknown; however, if the CLPs are factorial at an early stage in disease, they represent excellent therapeutic targets, not only biomarkers as currently proposed, not least because of the lack of expression in most normal tissues. Similarly in cancer, expression levels of CHI3L1 are correlated with poor prognosis,¹⁴ which could be due to inflammatory support of the tumor, mediated in part through the CLP. If, like in the mouse model presented here, these proteins prove to be primary autoantigens in human carcinogenesis, then targeting these proteins holds considerable therapeutic potential for several forms of cancer as well as a number of autoimmune, inflammatory disorders.

Materials and Methods

Transgenic mice. LMP1^{CAO} is derived from a nasopharyngeal carcinoma EBV strain. Two lines of L2LMP1^{CAO} transgenic mice (line 117 and line 105B) in a >99% FVB background were used in these studies.³⁰ Line 117 mice in RAG1-heterozygous or -null (RAG1^{-/-}) backgrounds have been previously described.³⁵ The transgene in line 117 is integrated into the Y chromosome, and as such, all females of this line are transgene negative and used as negative sibling controls (NSCs). Line 105B carries an autosomal transgene insert, and as such, transgenic and NSC are of both genders. All procedures have been conducted under UK Home Office license, and the research has complied with Home Office and institutional guidelines and policies. Tissue samples were snap frozen in liquid N₂ and stored at -70°C for sample extraction or formalin fixed at 4°C for immunohistochemical analyses. Serum was isolated by allowing blood to clot overnight and centrifuging at 14,000g for 10 minutes, and the supernatant was stored at -70°C.

Chemical carcinogenesis and cell lines. Transgenic and NSC mice were treated topically from 8 weeks old with a standard chemical carcinogen regime consisting of one dose of 25 µg dimethylbenzanthracene (DMBA), followed after 1 week by a 20-week biweekly treatment with 200 µL 5 × 10⁻⁵ M 12-O-tetradecanoylphorbol-13-acetate (TPA) as previously described.⁴² Cell lines were developed from carcinomas as described.⁴² Conditioned medium was collected from cells at confluence, clarified by centrifugation at 14,000g for 15 minutes, and stored at -70°C.

Immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at 2 µm, dewaxed, and subjected to pressure cooker antigen retrieval in 10 mM sodium

citrate, pH 6, buffer prior to incubation with primary antibody. Sections were stained using the appropriate secondary antibody conjugated to HRP and detected as per the manufacturer's instructions (K4010, EnVision+ system, DakoCytomation, Glostrup, Denmark). Following staining, all sections were washed in H₂O, counterstained with Gills hematoxylin, differentiated in 1% acid alcohol, and then the nuclei colored blue in Scott's tap water substitute. IHC primary antibodies were 1:500 rabbit anti-YM (DW) and 1:30 rat anti-Chi3L1 (MAB2649, R&D Systems, Minneapolis, MN). Images were captured using a Zeiss Axioskop 2 microscope (Oberkochen, Germany) and KS300i software (Imaging Associates, UK).

Immunoprecipitation (IP). Protein was extracted from ears by homogenization in NET-N buffer (150 mM NaCl, 5 mM EDTA, pH 8.0, 50 mM tris-HCl, pH 8.0, 0.05% NP-40) containing protease inhibitor cocktail (04906845001, Roche, Basel, Switzerland) and phosphatase inhibitor cocktail II (04906845001, Roche) and clarified by centrifugation at 16,000g for 15 minutes at 4°C and stored in aliquots at -70°C. Protein concentration was determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). YM IP and Chi3L1 IP were performed using 200 µg protein samples made up to 1 mL with NET-N with a preclear step and incubating the samples overnight with 30 µL of 50% protein G sepharose (PGS) at 4°C. This was followed by immunoprecipitation using 4 µL of anti-YM (DW) or 2 µL anti-Chi3L1 antibodies, incubated overnight, and captured with 30 µL of 50% PGS. To IP antibody-antigen complexes within the ear tissue ("self-IP"), 200 µg of protein extract (in 1 mL total volume) was rotated at 4°C overnight. There was 30 µL of 50% PGS used to capture the complexes. The immunoprecipitated complexes were washed twice with 1 mL of NET-N and 1 mL of PBS and eluted with 30 µL of either reducing (with 2-mercaptoethanol) or nonreducing (without 2-mercaptoethanol) loading buffer (7.5% [v/v] glycerol, 2.5% [v/v] 2-mercaptoethanol, 2% [w/v] SDS, 100 mM tris-HCl, pH 6.8, trace bromophenol blue) at 95°C and separated by 10% SDS-PAGE.

Western blotting. Proteins were extracted in RIPA buffer (150 mM NaCl, 50 mM tris-HCl, pH 7.5, 1% triton-X-100, 1% deoxycholic acid, 0.1% SDS) by polytron homogenization and sonication, using 10 to 50 µg per track as indicated. Serum samples (5 µL per track) were diluted with 25 µL RIPA buffer. Conditioned medium from cell culture (30 µL) was used undiluted. Reducing or nonreducing loading buffer (as above) was added to samples, which were separated by SDS-PAGE (10% gel). Blotting and washing were performed as previously described.³⁶ The blots were incubated in 5% nonfat milk PBS 0.1% (v/v) Tween 20 (Roche Applied Science, Mannheim, Germany) with the appropriate

antisera dilution. Antibodies used were 1:1,000 anti-YM (DW) or 1:1,000 anti-YM1 (AF2446, R&D Systems), 1:200 line 117 sera (combined from 4 transgenic mice or 4 NSC), anti-Chit1 (sc-46853, Santa Cruz Biotechnology, Santa Cruz, CA), anti-AMCase (sc-49355, Santa Cruz Biotechnology), anti-GAPDH (sc-32233, Santa Cruz Biotechnology), followed by either 1:4,000 goat anti-rabbit, goat anti-mouse, or donkey α -goat IgG HRP (sc-2030, sc-2031, sc-2033, Santa Cruz Biotechnology). Detection was performed by enhanced chemiluminescence-western detection kit (RPN 2209, GE Healthcare, Little Chalfont, UK).

RT-PCR. RNA was extracted and DNase treated from tissue as previously described.⁴³ For RT-PCR, first-strand cDNA was generated from the RNA samples by a kit (Abgene, Epsom, UK) according to the manufacturer's instructions, with or without (as control) reverse transcriptase. PCR was conducted using specific primers for YM1 (F: 5'CTGGAATTGGTGCCCCTACA3', R: 5'CAAGCATG GTGGTTTTACAGGA3'), YM2 (F: 5'CAGAACCGT CAGACATTCATTA3', R: 5'ATGGTCCTTCCAGTAGG TAATA3'), or GAPDH (F: 5'TCCACCACCCTGTTGCT GTA3', R: 5'ACCACAGTCCATGCCATCAC3') by first denaturing at 95°C for 3 to 5 minutes and then 30 to 35 cycles of 95°C for 30 seconds, YM1: 58°C, YM2: 50°C, GAPDH: 55°C for 30 seconds, 72°C for 30 seconds, and ending with 72°C for 10 minutes. Products were separated through 1% agarose gels in TAE buffer.

2-D DiGE proteomic analysis and YM identification by mass spectrometry. Full details of the 2-dimensional difference gel electrophoresis are described.³¹ Four replicates of each St2 and St5 transgenic ear tissue sample were compared with replicate NSC samples. Data from gels in the range of pH 4 to 7 are shown. For YM identification, 1 mg per sample of protein extracts from St2 and St5 and NSC tissues in 1 mL NET-N were precleared with 200 μ L PGS overnight and immunoprecipitated with 7 μ L anti-YM (DW). Washed (as above) immune complexes were separated by SDS-PAGE (comparing reducing and nonreducing conditions). Replicate gels were fixed for 3 hours (10% acetic acid, 40% ethanol), stained with 1:4 methylated spirits:colloidal Coomassie blue (10% (NH₄)₂SO₄, 1.2% orthophosphoric acid, 0.1% Coomassie blue) for 4 days, and destained for 3 days in H₂O. YM bands were cut out and stored in H₂O prior to mass spectrometry as described.³¹

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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