

Differential immunogenicity and allergenicity of native and recombinant human lactoferrins: Role of glycosylation

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Human native milk lactoferrin (LF) and recombinant forms of lactoferrin (rLF) are available with identical aa sequences, but different glycosylation patterns. Native lactoferrin (NLF) possesses the intrinsic ability to stimulate vigorous IgG and IgE antibody responses in BALB/c mice, whereas recombinant forms (*Aspergillus* or rice) are 40-fold less immunogenic and 200-fold less allergenic. Such differences are independent of endotoxin or iron content and the glycans do not contribute to epitope formation. A complex glycoprofile is observed for NLF, including sialic acid, fucose, mannose, and Lewis (Le)^x structures, whereas both rLF species display a simpler glycoprofile rich in mannose. Although Le^x type sugars play a Th2-type adjuvant role, endogenous expression of Le^x on NLF did not completely account for the more vigorous IgE responses it provoked. Furthermore, coadministration of rLF downregulated IgE and upregulated IgG2a antibody responses provoked by NLF, but was without effect on responses to unrelated peanut and chicken egg allergens. These results suggest glycans on rLF impact the induction phase to selectively inhibit IgE responses and that differential glycosylation patterns may impact on antigen uptake, processing and/or presentation, and the balance between Th1 and Th2 responses.

Keywords: Allergenicity · Glycosylation · Immunogenicity



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Introduction

Food (IgE-mediated) allergy is a significant public health issue in Westernized countries [1–3], with prevalence and/or awareness

increasing over the past five decades, particularly in children. Current estimates vary from 1 to 4% of adults and 5 to 8% of children [1–3]. There is a spectrum of activity among food proteins with respect to allergenic potential. Despite dietary exposure to thousands of immunogenic proteins, a narrow range of foods is responsible for the majority of food allergic reactions [4,5]. Thus, in silico analyses of plant (and pollen) allergens have demonstrated that more than 65% of plant allergens belong to four protein structural superfamilies [4].

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However, the introduction of novel foods into the national diet can result in changes in the pattern of food allergy. For example, following the relatively recent introduction of kiwi fruit into the UK diet, kiwis are now an increasingly important food allergen, causing severe reactions, particularly in young children [6]. Concern over the increased use of genetically modified food crops raises questions about associated health risks, including that the transgene products may have allergenic potential [7, 8].

It is important, therefore, to understand the characteristics that confer allergenicity on proteins, which may permit allergenic properties to be designed out, and have application in the development of therapeutic recombinant proteins, including mAbs. Various biochemical and physicochemical properties have been reported to be associated with allergenic activity, although none are exclusive to allergens [9, 10]. These include stability to heat and proteolytic digestion, the presence of repetitive structures, aggregate formation [9, 10], and for some allergens, proteolytic activity [11]. Glycosylation may also play a role in allergenicity and immunogenicity [9, 10, 12–14]. Sugar residues can act as IgE (and IgG) epitopes, particularly in certain plant-derived allergens [12–14] causing cross-reactivity between allergens, although such is not generally clinically relevant [12–14]. However, oligosaccharide-specific IgE can cause anaphylaxis, for example, following infusion of the mAb cetuximab [15]. Little attention has been paid to the role of carbohydrates in the induction of immune and allergic responses. Comparative studies using glycosylated native antigens (from *Schistosoma mansoni* [16, 17] or *Aspergillus fumigatus* [18]) have suggested an association between glycosylation and Th2-type adjuvant activity, with reduced type 2 responses observed following chemical deglycosylation.

In current investigations, we have utilized the availability of differentially glycosylated forms of human lactoferrin (LF) to examine the role of glycosylation in the induction of antibody responses. LF is an iron-binding protein with antimicrobial [19] and immunomodulatory properties [20], which has been identified as an alarmin [21]. Native lactoferrin (NLF) from human breast milk and recombinant forms (recombinant lactoferrin [rLF]) produced in *Aspergillus* and in rice have identical aa sequences [22, 23] but have different glycosylation profiles. The immunogenicity (IgG-inducing properties) and allergenicity (IgE-inducing potential) of the molecules was investigated in mice following i.p. exposure in the absence of adjuvant, a regimen shown previously to discriminate between allergens and non-allergens with respect to IgE production [24]. Herein, glycosylation was shown to play a critical role in the development of IgG and IgE antibody responses.

Results

Differential IgG and IgE antibody responses provoked by NLF and rLF

The immunogenicity and allergenicity of NLF and rLF were investigated. Initially, BALB/c strain mice were immunized i.p. with

1% of each protein; previous experience with a range of proteins indicating this should be sufficient to provoke immune responses [24]. Exposure to NLF resulted in vigorous anti-LF IgG antibody responses, whereas both recombinant materials provoked significantly lower titers when exposed to equivalent amounts of protein (Fig. 1A and B). Furthermore, NLF stimulated marked IgE responses (titers of 1 in 16 to 1 in 64) whereas both recombinant proteins at the same dose (1%) failed repeatedly to induce detectable IgE. Subclass analysis of the IgG response revealed that NLF induced more IgG1 antibody than rice rLF, although equivalent anti-LF IgG2a titers were recorded, thus ratios of IgG1:IgG2a specific antibodies were 1:0.3 and 1:2, respectively. Given the striking differential responses to NLF and rice rLF at equivalent immunizing concentrations (1%) (Fig. 1C and D), dose responses were conducted to explore further the relationship between the different forms of LF and immunogenicity/allergenicity, spanning concentrations from 5 to 0.025%. Immunization with the lower dose range of rLF (0.025–0.5%) failed to stimulate vigorous IgG or detectable IgE antibody responses (data not shown), whereas NLF induced detectable IgE and substantial IgG responses even at the lowest dose tested (0.025%). At the standard dose of rLF (1%), equivalent IgG titers were achieved to those induced by the lowest dose of NLF (0.025%). The number of IgE responders at each dose of NLF and rLF was analyzed (Fig. 1D). The majority of mice (nine or ten of ten) were high IgE responders after treatment with NLF (1–0.05%), although only 20% were IgE responders at the 0.025% dose. Mice treated with either 1% or 2% rice rLF were negative for specific IgE, with four of ten IgE responders recorded at the 5% dose. Thus, the native protein is approximately 40-fold more immunogenic and 200-fold more allergenic than the rice rLF. Differential clearance of the molecules did not appear to play a role. Within 6 h of injection of both NLF and rice rLF, the LF concentration in the peritoneum had reduced 1000-fold from the maximal levels recorded 2 h after injection (Supporting Information Fig. 1). The serum half-life also displayed very similar kinetics for both rLF and NLF, with high levels maintained in the serum for up to 4 h after initial injection, returning toward baseline after 6 h.

NLF and rLF exhibit differential glycosylation patterns

Given that the different forms of LF have identical aa sequences, the hypothesis that divergent glycosylation patterns contribute to the effects was examined. The monosaccharide and sialic acid content of NLF and rice rLF was quantified and compared with published data for the *Aspergillus* rLF (Table 1; [23]). The native form contained high levels of *N*-acetyl glucosamine (GlcNAc), mannose, galactose and also some fucose and, consistent with previously published data using carbohydrate electrophoresis technology [23], *N*-acetyl neuraminic acid (NeuAc; sialic acid). In contrast, rice rLF was more mannosylated (44% of sugars) with relatively high levels of fucose, GlcNAc, and xylose. *Aspergillus* rLF was highly mannosylated (87.7%), with GlcNAc the only other detectable monosaccharide (Table 1; [23]).

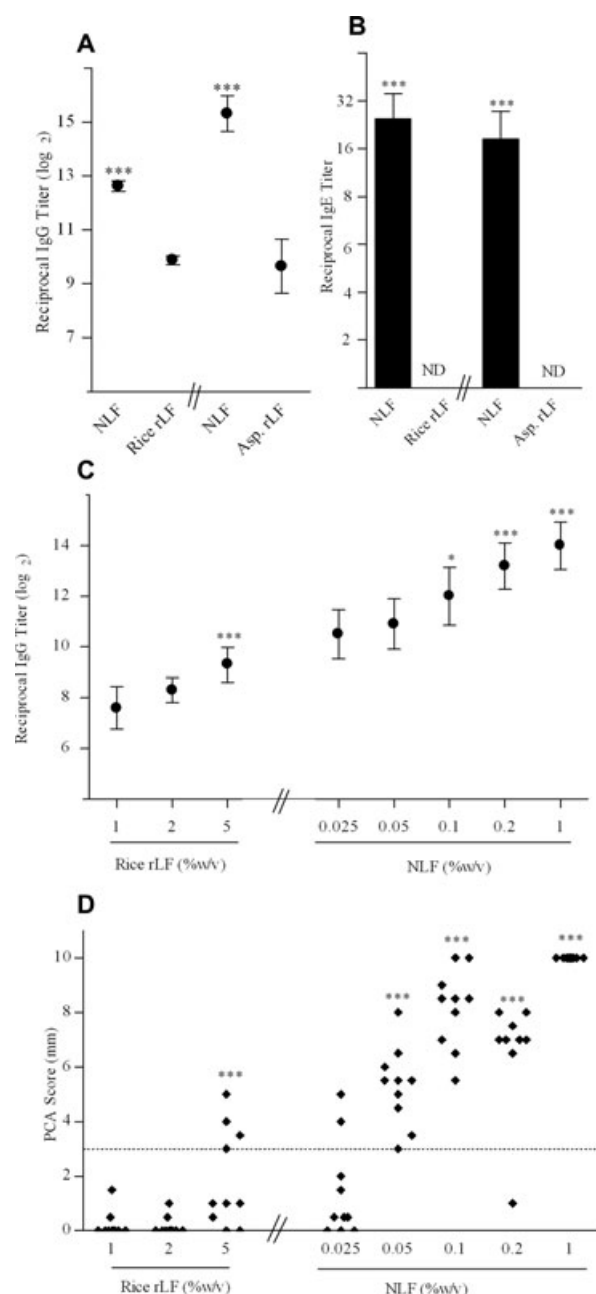


Figure 1. Differential IgG and IgE antibody responses to NLF and rLF. (A, B) Mice ($n = 10$ per treatment group per experiment) received NLF or rice or *Aspergillus* (Asp.) rLF by i.p. injection on days 0 and 7. Serum samples (day 14) were analyzed for specific IgG antibody by ELISA (individual serum samples) and IgE titer (pooled serum samples) by homologous PCA (using the immunizing LF as substrate). Data are shown as mean ($\pm$ SE) (A) reciprocal IgG ($\log_2$) and (B) IgE titer for eight independent experiments for rice rLF and three independent experiments for Asp. rLF. ND = IgE not detectable. (C, D) Mice received various doses of NLF (0.025–1%) or rice rLF (1–5%). Data are displayed as (C) the mean ($\pm$ SE) reciprocal IgG titer ($\log_2$) and (D) IgE responder frequency (PCA score for each individual serum sample) for one experiment, $n = 10$ mice per treatment group. Horizontal line = threshold (3 mm) for a positive PCA reaction. Significant differences between NLF versus rLF groups (A, B) and versus lowest LF dose (C, D) are indicated (one-way ANOVA, followed by Tukey's post hoc test for IgG; Student's t test for IgE titer; and Fisher's exact test for IgE responder frequency). * $p < 0.05$, *** $p < 0.001$.

Further enzymatic digestion of glycopeptides [25] (peptide:N-glycosidase (PNGase) F, endo- β -galactosidase and β -galactosidase, and sialidase for NLF; PNGase A and F for rice rLF) were analyzed by MALDI-TOF (Fig. 2 and Supporting Information Figs. 2 and 3). NLF exhibited a typical mammalian glycoprotein profile with core-fucosylated complex type glycans terminated with either NeuAc and/or Le^x (β -D-Gal-(1,4)-(α -L-Fuc-(1,3))- β -D-GlcNAc-R) epitopes (where Le is Lewis) (m/z 2605, 2779, and 2996). Many N -glycans on one of the antennae were extended with N -acetylglucosamine repeating units (m/z 3228, 3402, 3576, and 3852) substituted with fucose residues forming multiples of Le^x and/or Le^{y/b} epitopes. In addition to MALDI-TOF/TOF MS/MS (data not shown), the presence of Le^{y/b} terminal groups and multiples of Le^x moieties was confirmed by structures resistant to β -galactosidase and endo- β -galactosidase digestion, respectively, (Supporting Information Figs. 2 and 4) and GC-MS linkage analysis (Supporting Information Table 1). In comparison, rice rLF had a simpler glycomic profile. N -glycans released by PNGase F were mannose-rich structures, some of which exhibited xylose residues (m/z 1331, 1536, and 1822; Fig. 2B). Unlike mammalian N -glycan core-fucosylation that is exclusively α 1–6 linked and therefore can be released completely by PNGase F, the rice rLF may contain plant-specific α 1–3 linked core fucose residues that inhibit this enzyme. Therefore, an additional PNGase A digestion was employed for rice rLF. N -glycans released by this enzyme exhibited additional complex type core-fucosylated glycan structures (Fig. 2C). These structures were terminated in either GlcNAc (truncated; m/z 1750, 1781, and 1996) or Le^a residues (m/z 2129, 2374, and 2732).

All forms of LF have identical antibody-binding epitopes

Given the marked differences in glycosylation patterns, it was possible that antibody-binding epitopes were altered or masked [12–14]. Antisera raised against NLF or rice rLF proteins were therefore examined for reactivity against each of the two forms of LF as substrates (Fig. 3A and B). Sera raised against NLF, rice rLF, or *Aspergillus* rLF gave similar IgG binding (titer) and IgE reactivity (or lack of reactivity for both recombinant forms) regardless of which form of LF was used in the analysis.

The conservation of IgG-binding sites was confirmed by inhibition ELISA. The ability of soluble NLF and rice rLF to inhibit binding of antisera raised against both NLF and rice rLF to plate-bound NLF was examined (Fig. 3C and D). Preincubation of LF antisera with the unrelated protein OVA was without effect. However, binding of NLF or rice rLF antisera to immobilized NLF was inhibited very effectively by soluble NLF and rice rLF. Thus, IC₅₀ values (concentration required for 50% inhibition of binding) of 10 ± 2 and 10 ± 1 μ g/mL were recorded with rice rLF antisera, and of 10 ± 5 and 9 ± 5 μ g/mL (mean $\pm$ SE, $n = 3$) with NLF antisera, following incubation with soluble NLF and rice rLF respectively.

Table 1. Monosaccharide composition of lactoferrins^{a)}

Glycan analyzed	Native human milk lactoferrin		Rice recombinant lactoferrin		A. awamori recombinant lactoferrin ^{b)}
	Total carbohydrate (nmol/mg)	Percentage of total carbohydrate	Total carbohydrate (nmol/mg)	Percentage of total carbohydrate	Percentage of total carbohydrate
Arabinose	0.0	0.0	10.4	4.5	0.0
Rhamnose	0.0	0.0	0.5	0.2	0.0
Fucose	40.9	13.7	31.3	13.5	0.0
Xylose	0.3	0.1	36.3	15.7	N/A ^{c)}
Mannose	84.1	28.2	102.0	44.1	87.7
Galactose	67.2	22.5	12.4	5.4	0.0
Glucose	1.6	0.5	3.5	1.5	N/A
GalNAc	0.6	0.2	0.0	0.0	0.0
GlcNAc	83.2	27.9	34.8	15.1	12.3
NeuAc	20.8	7.0	0.0	0.0	0.0
NeuGc	0.0	0.0	0.0	0.0	0.0
Total	298.6	100	231.2	100	100

^{a)}Monosaccharide content analysis, expressed as nano moles of sugar per milligram of rice rLF or NLF and as percentage of sugar content, was carried out by GC-MS and for sialic acid analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

^{b)}For comparative purposes, published data for monosaccharide content (%) of *Aspergillus* rLF are shown. This glycoprofile has recently been reanalyzed by Agennix AG and displays some differences from that published previously (personal communication; Dr. E. Quackenbush, Agennix AG). However, mannose remains the most highly expressed residue.

^{c)}N/A: glycans not analyzed.

GalNAc: N-acetylgalactosamine; GlcNAc: N-acetyl glucosamine; NeuAc: N-acetyl neuraminic acid; NeuGc: N-glycolylneuraminic acid.

Effect of glycosylation on initiation of immune responses

Having demonstrated that it is unlikely that the carbohydrate groups make a major contribution to antibody-binding epitopes, their effect on the induction of antibody responses was examined. Attempts to remove the sugar residues chemically using trifluoromethylsulfonate [26] were unsuccessful due to protein denaturation (data not shown). Instead, the ability of concurrent administration of rice rLF (1%) to modulate specific antibody responses to a submaximal dose of NLF (0.2%) was investigated. Immunization with NLF alone resulted in detectable IgE antibody in the majority of recipients (nine of ten IgE responders), whereas rice rLF failed to provoke IgE (zero of ten responders) (Fig. 4A). Although the majority (eight of ten) of mice immunized with the mixture of NLF/rice rLF were IgE responders, the mean passive cutaneous anaphylaxis (PCA) scores for these mice were significantly ($p < 0.01$) reduced compared with mice immunized with NLF alone. This difference was reflected in the IgE titers, with titers of 1/8, 1/16, and 1/32 recorded following immunization with NLF alone compared with a titer of 1 recorded for the LF mixture (Fig. 4B). Furthermore, exposure to rice rLF influenced the IgG subclass distribution of the response (Fig. 4C). Thus, rice rLF provoked significantly higher levels of IgG2a antibody than did NLF, with increased IgG2a antibody recorded for the NLF/rice rLF mixture. The ability of rice rLF to inhibit IgE antibody responses was antigen specific; coadministration of rice rLF with 0.2% OVA or peanut lectin did not impact induced IgE titers (titers of 1 in 4 and 1 in 16, respectively, were observed regardless of the presence

of 1% rice rLF). It was necessary for exposure to be concomitant for the effect to be observed; priming with rLF did not inhibit subsequent responses to NLF (data not shown).

The ability of Le^x sugar components to act as adjuvants was also examined. Exogenous Le^x was added in a 1:10 ratio to rice rLF and IgE responses measured (Fig. 4D). Concurrent administration of Le^x resulted in increased IgE compared with rice rLF alone (titers of 1 in 2 to 1 in 8 versus titers of 1 (undiluted serum) or 1 in 2), however, such did not approach the levels observed for NLF.

Impact of LF on LC migration and DC activation

The ability of all three forms of LF to inhibit Langerhans' cell (LC) migration was investigated (Fig. 5). This experimental approach confirmed biological activity and demonstrated that all forms of LF possessed equivalent immunomodulatory function. LC migration requires cytokine signals from IL-1 β and TNF- α and is compromised by *Aspergillus* rLF application due to inhibition of local TNF- α production [22, 27]. Resting LC frequency in the skin of naïve female BALB/c mice was approximately 1500 LC/mm²; topical exposure to the contact allergen oxazolone (4 h) resulted in a significant loss of LC (~30%; the proportion of LC that is able to migrate rapidly from the epidermis). Prior topical application of NLF, rice rLF, or *Aspergillus* rLF each abrogated the ability of oxazolone to stimulate a significant reduction in LC frequency (Fig. 5). For the rice rLF, this effect was due to inhibition of TNF- α ; thus prior exposure to rice rLF did not inhibit LC migration induced by exogenous TNF- α , but significantly

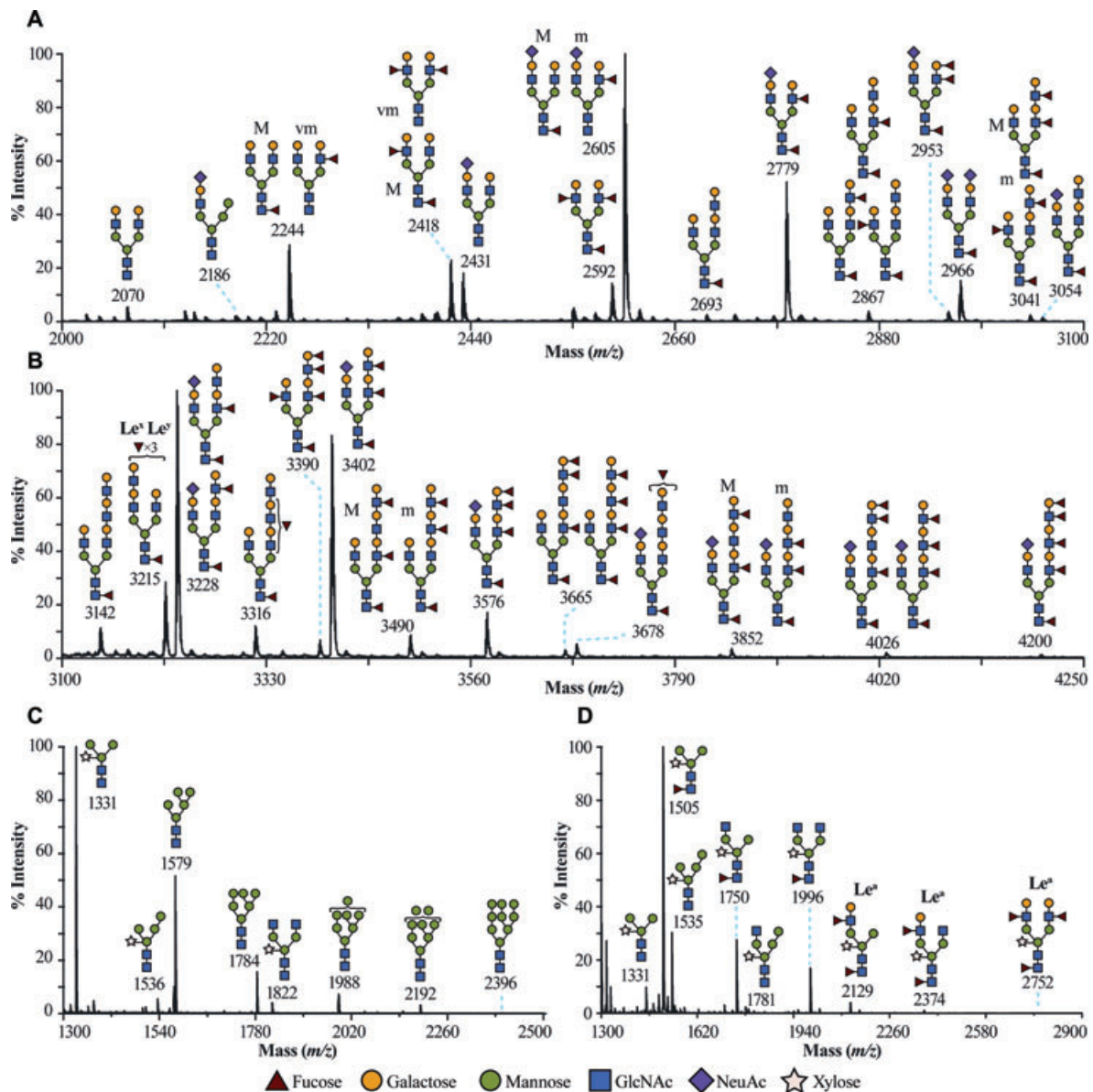


Figure 2. Glycosylation profiles of LF. MALDI-TOF MS of permethylated N-glycans from (A, B) NLF after PNGase F digestion (A, 2000–3100 m/z and B, 3100–4250 m/z) and (C, D) rice rLF after (C) PNGase F and (D) PNGase A digestions. N-glycomic profiles were obtained from the 50% MeCN fraction from a C_{18} Sep-Pak column. Schematic structures are according to the Consortium for Functional Glycomics guidelines (www.functionalglycomics.org). All molecular ions are $(M+Na)^+$. Putative structures are based on composition, tandem MS, and biosynthetic knowledge. Bracketed structures have not been unequivocally defined. “M,” “m,” and “vm” correspond to major, minor, and very minor abundant structures. Data shown are a summation of spectra from 5000 shots of the MALDI laser.

inhibited migration induced by exogenous rIL-1 β (which requires endogenous TNF- α). Finally, the impact of NLF and rice rLF on DC activation was investigated. BMDCs were cultured on day 8 for 24 h with various concentrations (1–100 μ g/mL) of LF and DC activation assessed as a function of changes in membrane marker expression (Supporting Information Fig. 5). The NLF caused a substantial and dose-dependent upregulation of MHC class II (MHC II) and the costimulatory molecule CD86, whereas rice rLF had little effect on these markers over the same concentration range. Interestingly, neither molecule influenced markedly CD80 expression.

Discussion

The current investigations have demonstrated that glycosylation influences the development of IgG and IgE antibody responses; rLF was 40-fold less immunogenic and at least 200-fold less allergenic than NLF. The IgE-inducing NLF exhibited a complex glycoprofile with highly fucosylated branched chains containing many Le^x trisaccharides, consistent with a report characterizing NLF and rLF produced in transgenic cattle [28]. The monosaccharide composition of NLF was reported to be 34% GlcNAc, 26% mannose, and 16% galactose and fucose with 8% NeuAc. Furthermore, NLF

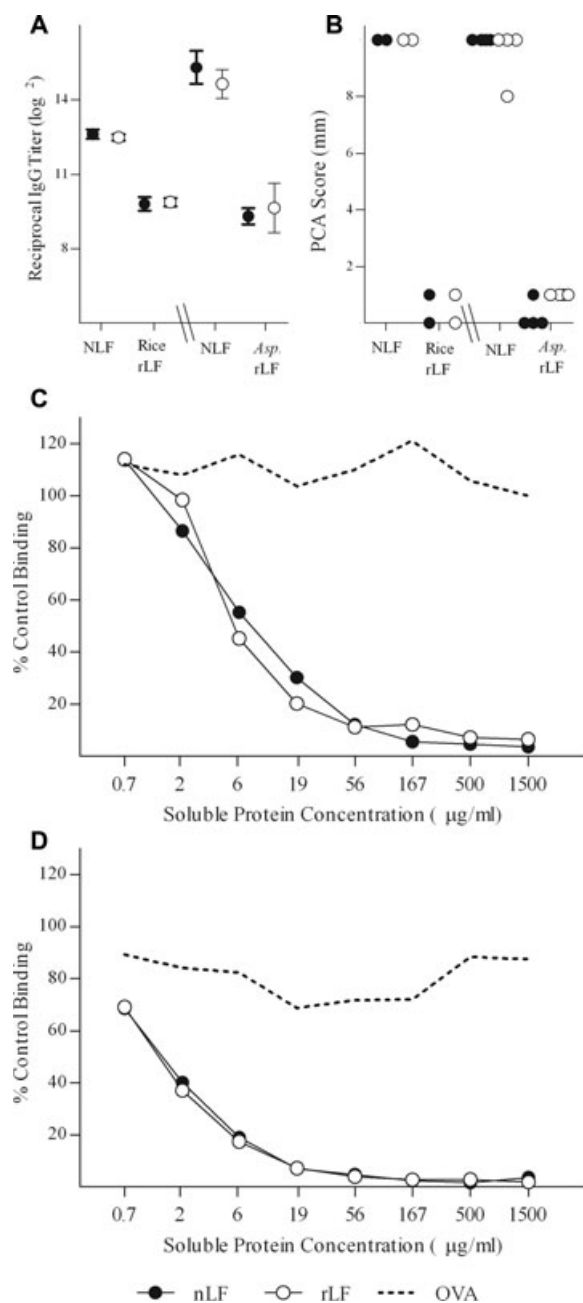


Figure 3. Cross reactivity of anti-LF antisera. (A, B) Mice ($n = 5$ per treatment group per experiment) received 1% of NLF or rLF (rice or *Aspergillus* (Asp.)) by i.p. injection on days 0 and 7. Serum samples (day 14) were prepared and cross-reactivity of (A) IgG and (B) IgE antibody responses measured by ELISA (individual serum samples) and by homologous PCA (pooled serum samples), respectively, using both NLF and rLF as substrates. Data are displayed as the mean ($\pm$ SE) reciprocal log₂ IgG titer (pooled from 3 independent experiments in A) and PCA score in two or four recipient animals for IgE responder status (one experiment representative of three performed in B). Horizontal line = threshold (3 mm) for a positive PCA reaction. (C, D) Cross-reactivity of the IgG antibody responses was investigated by inhibition ELISA. Inhibition of binding of (C) NLF antisera (diluted one in 200) and (D) rice rLF antisera (diluted one in 50) to solid-phase NLF by soluble NLF, soluble rice rLF, or the irrelevant allergen OVA was measured. Anti-LF IgG binding was determined using anti-mouse IgG HRP-labeled detection antibody. Data are shown as percentage of control binding (binding in the absence of soluble antigen) for one experiment representative of three performed.

was also shown to be abundant in Le^x moieties. Interestingly, despite production in mammalian cells the glycoprofile of the bovine rLF was heavily mannosylated (45%), with high levels of GlcNAc (30%), a pattern more similar to that recorded for the rice and *Aspergillus* proteins. The speculation is, therefore, that bovine rLF lacks the ability to provoke vigorous IgE antibody responses in mice. Indeed, WT bovine LF, which also presumably lacks the complex glycoprofile of human LF, failed to provoke vigorous IgE responses (data not shown).

The carbohydrate groups were not involved in and did not influence the epitope-binding sites, despite previous reports that glycans including mannose, xylose, and fucose linked sugars can act as IgE recognition motifs [14]. Various forms of LF have been shown to be immunomodulatory, including affecting cytokine production [20, 21, 23]. NLF has previously been shown to inhibit epidermal LC migration [22]. Despite their differential glycosylation and IgE-inducing properties, all three forms of LF displayed equivalent potential to inhibit the mobilization and migration of LC. Thus, differential glycosylation does not impact this known biological activity of LF. However, the sugars are influencing the induction of immune and allergic responses to LF, presumably at the level of antigen uptake, processing, and/or presentation by DCs. Indeed, NLF was more effective than rice rLF at causing the in vitro maturation of BMDCs with respect to membrane expression of the costimulatory molecule CD86 and upregulation of MHC II.

We hypothesized that the Le^x moiety on the NLF is responsible for the IgE-inducing properties of this molecule. This oligosaccharide is an isomer of the blood group antigen Le^a and is also expressed in pentameric form (lacto-*N*-fucopentaose III [LNFPIII]) in breast milk [29] and in oligosaccharide structures of *S. mansoni* glycolipids [17, 30]. When covalently linked to HSA, LNFPIII stimulated vigorous Th2 responses in BALB/c mice, causing increases in total IgE and specific IgG1 and IgE antibody, with the conjugate being 1000-fold more potent at stimulating antibody production than was unconjugated human serum albumin (HSA) [17]. In those studies, free oligosaccharide was without effect and the sugar residues were not involved in the antibody-binding sites. There are also reports that LNFPIII is a potent inducer of IFN- γ in vitro, indicating the potential of this molecule to exert opposing influences on the quality of induced immune responses [17]. The type 1 inducing effects are apparently TLR-mediated whereas the in vivo Th2-type adjuvant properties of Le^x have been shown to be LPS-TLR4 independent [31]. Thus, Le^x-BSA conjugates stimulated more vigorous IgE responses and a more Th2 polarized cytokine profile than did BSA alone. Furthermore, coadministration of free Le^x with OVA (in a 1:10 ratio) enhanced anti-OVA IgE-antibody responses to an equivalent extent to that induced by the archetypal Th2 adjuvant alum [31].

Immunization of mice with rice rLF in the presence of free Le^x resulted in increased IgE titers, however, such did not match the vigor of anti-NLF IgE responses. Although it could be argued that the free material is less effective than the conjugated material, the ratio of free Le^x to protein (1:10) is at least 10-fold in excess to that expressed by WT NLF. Furthermore, when mice were immunized with a mixture of NLF and rLF, IgE responses to the NLF were

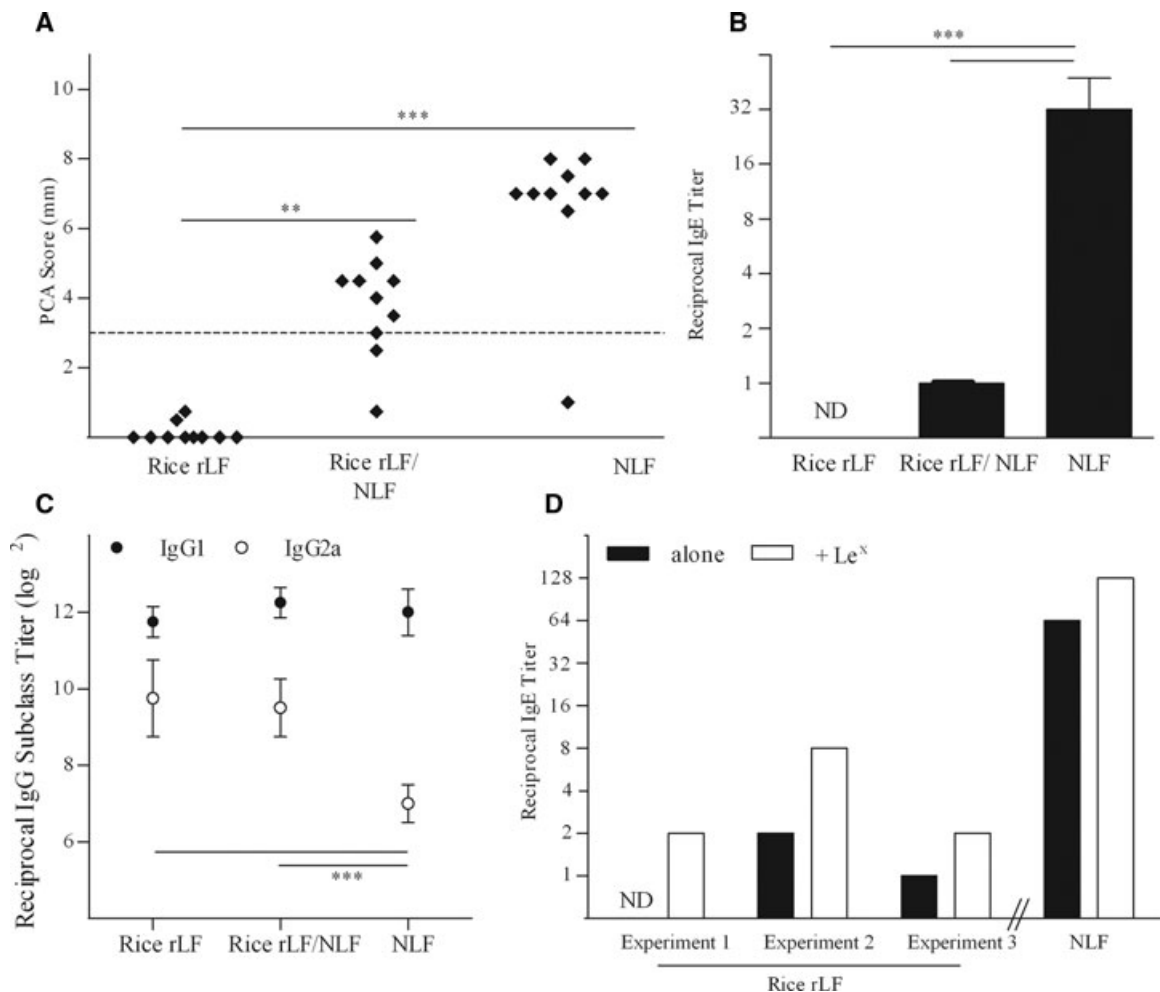


Figure 4. Influence of coadministration of rice rLF and LF and of exogenous Le^x. (A, B, C) Mice ($n = 10$ per treatment group per experiment) received 0.2% NLF or 1% rice rLF alone or in combination by i.p. injection on days 0 and 7. Serum samples (day 14) were analyzed for (A) IgE responder frequency (PCA score; individual serum samples from one experiment representative of three performed), (B) IgE titer by homologous PCA (mean $\pm$ SE titer of pooled serum samples from three independent experiments), and (C) IgG1 and IgG2a antibody by ELISA (mean $\pm$ SE ($\log_2$) titer of individual serum samples from one experiment representative of three performed). In each case, NLF was used as substrate. Horizontal line = threshold (3 mm) for a positive PCA reaction. ND = IgE not detectable. Significant differences between groups are indicated (one-way ANOVA, followed by Tukey's post hoc test for IgG1 and IgG2a titer, Student's t test for IgE titer, and Fisher's exact test for IgE responders). ** $p < 0.01$; *** $p < 0.001$. (D) Additional groups of mice ($n = 5$ per treatment group) received 500 μ g of rice rLF (in three independent experiments) or NLF alone or in combination with 50 μ g of Le^x (representative from one of the three independent experiments) by i.p. injection on days 0, 7, and 14. Twenty-one days after initiation of exposure, pooled serum samples were prepared and analyzed for the presence of specific IgE by homologous PCA, using NLF as substrate. Data are displayed as reciprocal titer for each individual experiment.

profoundly downregulated. These data suggest that in addition to the Le^x moieties on the NLF having IgE- and IgG-inducing properties, the glycan groups expressed by the recombinant proteins have suppressive effects. The recombinant proteins that lack IgE-inducing properties express considerably higher levels of mannose than the WT protein, and are also relatively rich in the expression of GlcNAc. Both carbohydrates and Le^x have affinity for the mannose receptor (MR), which possesses multiple carbohydrate recognition domains and is expressed primarily by macrophages and DCs [32]. Uptake of a number of antigens through the MR has been shown to be associated with Th1 responses [33–38]. For example, when uptake of *Candida albans* was manipulated by blocking the MR and opsonization of the bacteria to target Fc γ receptors, type

2 responses and pathology were observed [34]. Activation of the MR was shown to result in increased IL-12 production by both murine and human DCs independently of TLR signaling [34, 35]. There are conflicting reports that suggest that glycosylated allergens such as *Der p 1* and *Can f 1*, which are also rich in mannose, may be taken up through the MR of human monocyte-derived DCs isolated from house dust mite sensitive (and atopic) individuals [36]. For *Der p 1*, it was shown that activation of MR-deficient DCs with allergen and LPS polarized autologous T cells down a Th1, rather than Th2, pathway [36]. The fact that activation through the MR may result in both Th1 and Th2 responses may be dependent on the nature of the allergen, the role of LPS coactivation and also the source of DCs. The Th1-type antibody profile provoked in

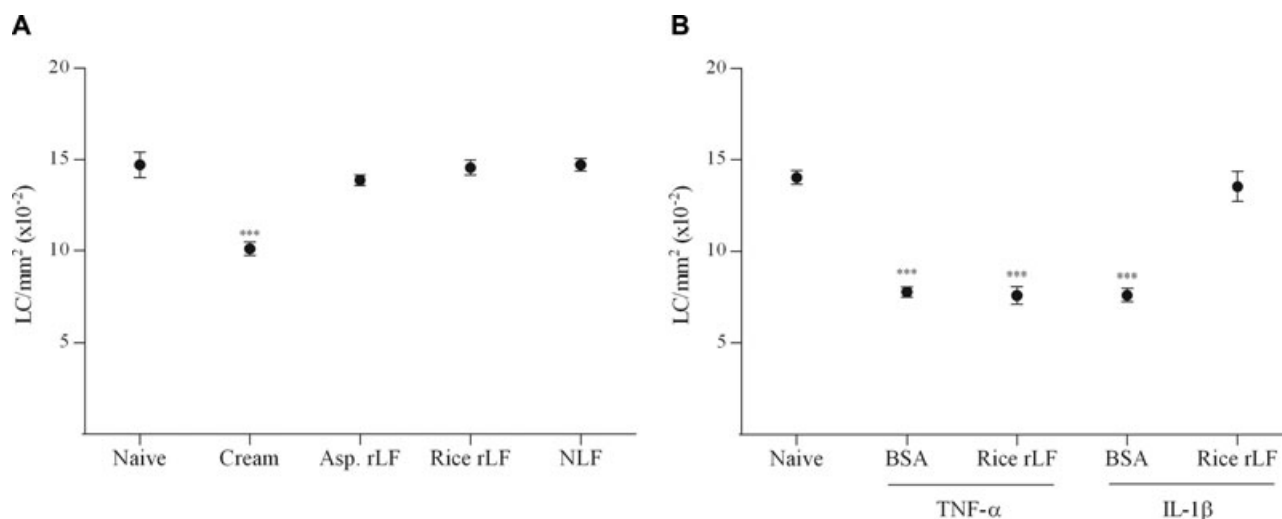


Figure 5. Effect of LF on LC migration. Groups of mice ($n = 3/\text{group}$) received 30 μL of NLF, rice rLF, *Aspergillus* (Asp.) rLF, or BSA (0.5 μg) in cream or cream alone on the dorsum of both ears. Two hours later mice received (A) a topical application of 0.5% oxazolone in acetone:olive oil (AOO) or (B) 50 ng of either IL-1 β or TNF- α by intradermal injection into the dorsum of both ears. Control mice were untreated (naive). Ears ($n = 6/\text{group}$) were removed either 4 h (A), 30 min (B; TNF- α), or 2 h (B; IL-1 β) after challenge and epidermal sheets prepared for analysis of LC frequency. Data are displayed as mean ($\pm\text{SE}$) LC frequency per treatment group for one experiment representative of three performed. Statistical significance of changes in LC frequency versus naive controls (by one-way ANOVA, followed by Tukey's post hoc test), *** $p < 0.001$.

BALB/c strain mice by rice rLF (little or no IgE; relatively vigorous IgG2a antibody response compared with IgG1) independent of LPS is consistent with Th1 activation via the MR [33–35]. There are also reports that mannose-type glycans can reduce the overall immunogenicity of recombinant glycoproteins [37, 38].

It should be noted that the MR is not the only DC receptor that binds mannose; others include DC-SIGN, which has high affinity for mannose and fucose and also binds Le^x [39]. This represents a potential mechanism by which coadministration of rice rLF with NLF downregulated profoundly IgE responses to NLF but was without effect on irrelevant allergens. The marked inhibition of IgE and the partial reduction in IgG is consistent with the induction of Th1-type responses, given that Th1 cytokines are not permissive for IgE and that Th1 cells are less effective at providing help for antibody responses [40]. Binding of different carbohydrate groups to DC-SIGN can trigger different patterns of intracellular signaling and activation [41]. Thus, mannose- and fucose (Le^x)-containing ligands recruited different intracellular scaffold proteins resulting in the expression of IL-10, IL-12, and IL-6 or the downregulation of IL-12 and enhanced production of IL-10, respectively. Successful displacement of Le^x-rich NLF from DC-SIGN by mannose-containing rice rLF could therefore upregulate IL-12 expression and enhance Th1-type responses while inhibiting IgE antibody production. Alternatively, it may be that differential receptor binding (to the MR or to DC-SIGN) could target allergen to different endosomal compartments and hence differential antigen presentation. A precedent for this is antigen targeted to the DEC-205 endocytic receptor being recycled to late endosomal/lysosomal vacuoles rich in MHC II for efficient presentation whereas the same antigen bound to the MR was recycled to early endosomal compartments and interacted less effectively with T cells [42].

In conclusion, marked differences in immunogenicity and allergenicity have been demonstrated among proteins with identical aa sequences but differential glycan profiles. Proteins with high levels of mannose lacked allergenicity and displayed a Th1-type antibody profile whereas Le^x expression was associated with vigorous IgE antibody responses; such differences are hypothesized to be due to differential uptake and processing by APC. These findings have important implications for the design and development of recombinant proteins; manipulation of the glycan profile may facilitate the design of adjuvants or hypoallergenic proteins.

Materials and methods

Mice

Female (8- to 12-week old) BALB/c strain mice (SPFU, Alderley Park, Cheshire, UK or Harlan Seralab, Loughborough, Leicestershire, UK) were used. Food and water were available ad libitum (Special Diet Services Rat and Mouse No. 1 Maintenance Diet; Special Diets Services, Witham, Essex, UK). All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 under a Home Office approved project licence.

Test materials

NLF (isolated from human breast milk, 90% pure; Sigma St Louis, MO, USA), rLF expressed in rice (*Oryza sativa*; 90% pure; Seracare Life Sciences Milford, MA, USA), or in *Aspergillus niger* var. *awamori* (95% pure; Agennix, Houston, TX, USA) as previously

described [23] were used in these studies. Endotoxin content was determined by quantitative limulus amebocyte assay according to the manufacturer's instructions (Cambrex BioSciences, Wokingham, UK).

Monosaccharide content of NLF and rLF

For monosaccharide content analysis, 50 μ L of NLF (488.4 μ g) and rice rLF (522.5 μ g) in 5% acetic acid were spiked with a standard (10 μ g arabinol) and lyophilized. These samples, an arabinol reagent blank (10 μ g) and standard mixtures of known amounts of reference monosaccharides, were incubated for 16 h at 80°C with 200 μ L of methanolic HCl. Lyophilized products were incubated sequentially for 15 min at room temperature with a mixture of ethanol (500 μ L), methanol, pyridine (10 μ L), and acetic anhydride (50 μ L) for re-*N*-acetylation and trimethylsilylated using Tri-sil Z (100 μ L; Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra and retention time were analyzed using a Perkin Elmer Turbo GC-MS system (Perkin Elmer, Waltham, MA, USA) and a DB-5 GC column (J&W Scientific, Folsom, CA, USA).

For the quantification of sialic acid, 50 μ L aliquots of NLF and rice rLF were spiked with the standard 2-keto-3-deoxynonulosonic acid and then lyophilized. Samples were hydrolyzed (0.1 M HCl for 1 h at 80°C), purified using dowex column chromatography (50 W-X8; VWR International, West Chester, PA, USA), eluted with 5% acetic acid and Speed-Vac reduced followed by lyophilization. These samples were resuspended and analyzed using Dionex high performance anion exchange chromatography with pulsed amperometric detection (ICS3000 system Chromeleon version 6.80; Dionex, Sunnyvale, CA, USA).

N-glycomic profile of NLF and rice rLF

The *N*-glycomic profiles were determined according to Sutton-Smith and Dell [25]. Briefly, samples were subjected to reduction in 4 M guanidine HCl, carboxymethylation, dialysis, and trypsin digestion. The digested glycoproteins were purified using Sep-Pak purification (Sep-Pak C₁₈ cartridge; Waters Corp., Hertfordshire, UK). *N*-Glycans on NLF were released by PNGase F (Roche Applied Sciences, Penzberg, Germany) digestion, while the rice rLF was additionally incubated with PNGase A (Roche). *N*-glycans were then permethylated using the sodium hydroxide procedure, and purified by C₁₈-Sep-Pak. MS and MS/MS data were acquired using a 4800 MALDI TOF/TOF (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) mass spectrometer. For MS/MS, the collision energy was set to 1 kV, and argon was used as collision gas. The 4700 calibration standard kit (Calmix; Applied Biosystems) and human (Glu1) fibrinopeptide B (Sigma) were used as external calibrants for the MS and MS/MS modes, respectively. The data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glycobioinformatics tool, GlycoWorkBench [43]. Selected peaks were assigned on the basis of

¹²C isotopic composition and knowledge of the biosynthetic pathways and confirmed by MS/MS.

Glycosidase digestions

β -galactosidase (bovine testes; Prozyme, Europa Bioproducts LTD, Cambridge, UK) 24 h digestion was performed in 200 μ L of ammonium acetate (37°C, 50 mM, pH 4.6) by addition of 10 mU of the enzyme at 0 and 12 h. Sialidase (*Vibrio cholera*; Roche) 24 h digestion was carried out in 200 μ L of sodium acetate (37°C, 50 mM, pH 5.5) by addition of 50 mU of the enzyme at 0 and 12 h. Endo- β -galactosidase (*Escherichia freundii*, Seikagaku Corp., Japan) 48 h digestion was carried out in 200 μ L of sodium acetate buffer (37°C, 50 mM, pH 5.8) by addition of 20 mU of the enzyme at 0 and 24 h.

Partially methylated alditol acetates

Linkages of the *N*-glycans of NLF were determined with partially methylated alditol acetates that were prepared as described previously [25]. Analysis was performed on a PerkinElmer Clarus 500 instrument fitted with a RTX-5 fused silica capillary column (Restek Corp., Bellefonte, PA, USA).

Dendritic cell culture

BM-derived DCs were generated as described previously [44]. Cells were isolated from the femurs and tibias of BALB/c mice and cultured at 2×10^6 cells/mL in culture medium supplemented with GM-CSF (Peprotech, London, UK; 20 ng/mL) for 8 days. Cells were resuspended at 10^6 cells/mL and cultured in the presence of 1–100 μ g/mL of NLF or rice rLF or medium alone for 24 h. Analysis of membrane marker expression was conducted by flow cytometry [42].

LC migration

Groups of mice ($n = 3$) received 30 μ L of 0.5 μ g LF in aqueous cream BP (Boots, Nottingham, UK) or cream alone topically on the dorsum of both ears. Two hours later mice were exposed to 30 μ L of 0.5% 4-ethoxy-2-phenyloxazol-5-one (oxazolone; Sigma) dissolved in acetone:olive oil (4:1). Four hours later mice (including naïve mice) were terminated and epidermal sheets prepared. For cytokine-induced migration, mice were exposed to LF or BSA (Sigma) in cream as previously. Ninety minutes later mice received a 30 μ L intradermal injection into both ear pinnae of recombinant TNF- α or IL-1 β (specific activity $1\text{--}2 \times 10^8$; R&D Systems, Minneapolis, MN, USA) in 0.1% BSA or BSA alone. Mice were terminated (TNF- α ; 30 min later or IL-1 β ; 4 h later) and epidermal sheets prepared as described previously [22]. Sheets were incubated with anti-mouse Ia/Ie FITC antibody (BD Biosciences,

San Jose, CA, UK), diluted 1:100 in 0.1% BSA for 1 h at room temperature, and then mounted on microscope slides in citifluor (Citifluor Ltd, London, UK). The frequency of LC was assessed by fluorescence microscopy using an eyepiece with a calibrated grid (0.32 mm × 0.213 mm at ×40 magnification; ten random fields per sample).

Sensitization for antibody responses

Groups of mice ($n = 5$ or 10) received 0.25 mL of protein (LF, OVA (Sigma; 96% pure) or peanut lectin from *Arachis Hypogaea* (98% pure; Sigma)) in PBS by i.p. injection on days 0 and 7. In some experiments, mice received a mixture of rice rLF (1%) with 0.2% NLF, OVA, or peanut lectin using the same dosing regimen. For adjuvant (Le^x; Dextra Laboratories, Reading, Berkshire, UK) experiments, mice were immunized i.p. with 0.25 mL of 500 µg of protein with or without 50 µg/mL of Le^x on days 0, 7, and 14. Fourteen or 21 days after the initiation of exposure, all mice were exsanguinated. Serum samples were prepared and stored at −70°C.

Measurement of LF half-life

The half-life of LF in the serum and peritoneal cavity was measured using a capture ELISA specific for human LF. Mice ($n = 4$) received a single i.p. injection of 1% LF and at various times thereafter a 5 mL PBS peritoneal wash was obtained followed by serum collection. Plastic microtiter plates (Nunc, Copenhagen, Denmark) were coated with 0.1 µg/mL of monoclonal anti-LF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and then blocked with 2% BSA/PBS at 37°C for 30 min. Samples diluted to various extents in 1% BSA/PBS or a standard curve of serially diluted LF were added and plates incubated for 6 h at room temperature. Following addition of biotinylated rabbit anti-LF (one in 10 000 dilution; Abcam, Cambridge, UK) and overnight incubation at 4°C, Extravidin-HRP (one in 1000; Sigma) was added and plates incubated for 2 h at room temperature. Enzyme substrate (3,3',5,5'-tetramethylbenzidine) was added and the reaction stopped with 2 M sulfuric acid. Substrate conversion at OD 450 nm was measured using an automated reader.

Measurement of anti protein IgG, IgG1, and IgG2a antibody responses

Protein-specific IgG, IgG1, and IgG2a antibodies were detected by ELISA. Plastic microtiter plates were coated with 100 µg/mL of protein in PBS overnight at 4°C and then blocked with 2% BSA/PBS at 37°C for 30 min. Serial doubling dilutions of serum samples in 1% BSA/PBS were added and plates were incubated for 3 h at 4°C. Plates were incubated for 2 h at 4°C with HRP labeled sheep anti-mouse IgG (one in 4000), rat anti-mouse IgG1 (one in 1000), or rat anti-mouse IgG2a (one in 2000) antibody; all anti-

bodies are from Serotec, Kidlington, Oxfordshire, UK. Enzyme substrate (*o*-phenylenediamine) was added and the reaction stopped with 0.5 M citric acid. Substrate conversion at OD 450 nm was measured using an automated reader. Titer was determined as the maximum dilution of serum at which an OD 450 nm reading of greater than 0.5 was achieved.

IgG inhibition ELISA

Plates were coated with LF as described above. Equal volumes of diluted serum (anti-NLF antisera, 1/200; anti-rice rLF antisera, one in 100) and various concentrations of protein (LF or irrelevant protein OVA; final concentrations from 0 to 1500 µg/mL) diluted in 10% FCS/PBS were admixed and incubated for 30 min at room temperature before addition to the plate and incubation at room temperature for 3 h. Reagent blank wells received 10% FCS/PBS alone. Detection antibody and substrate steps were as described above. Percentage inhibition by soluble protein of binding was calculated after subtraction of reagent blank values.

Measurement of anti-protein IgE antibody responses

Protein-specific IgE antibodies were detected by PCA assay as described previously [24]. For IgE responder frequency, individual (undiluted) serum samples were analyzed ($n = 2$ recipients). IgE titers were determined using pooled serum samples injected (30 µL) into the dermis of the ears of naïve recipient mice ($n = 3$ – 4). Two days later, 0.25 mg of relevant protein and 1.25 mg of Evans blue dye (Sigma) were injected i.v. Thirty minutes later, the diameter of the cutaneous reaction was measured. A positive (IgE) response was recorded if a > 3 mm reaction was recorded in the majority of recipient animals (pooled samples), with antibody titer recorded as the highest dilution resulting in a positive PCA reaction. For individual serum samples, the mean diameter of the cutaneous reaction in recipient animals was recorded, and the sample was identified as an IgE responder if the mean diameter of the PCA reaction was >3 mm.

Statistical analyses

Direct comparisons between responses to NLF and rLF, IgG, IgG1, IgG2a, and IgE antibody titers were analyzed following logarithmic transformation ($\log_2$) by Student's *t* test for direct comparisons (no detectable IgE was assigned the value zero posttransformation) or for multiple immunization groups, and for changes in LC frequency (not transformed) by one-way ANOVA followed by Tukey's post hoc test. IgE responder and nonresponder frequency was compared using contingency tables (Fishers exact). All statistical tests were carried out using GraphPad Prism (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; GraphPad, San Diego, CA, USA).

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Abbreviations: LNFPIII: lacto-N-fucopentaose III · LC: Langerhans cell · Le: Lewis · LF: lactoferrin · MR: mannose receptor · GlcNAc: N-acetyl glucosamine · NeuAc: N-acetyl neuraminic acid · NLF: native LF · PCA: passive cutaneous anaphylaxis · PNGase: peptide:N-glycosidase · rLF: recombinant LF

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