

Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses

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Immunoglobulin (Ig)-free light chains IgLC are present in serum and their production is augmented under pathological conditions such as multiple sclerosis, rheumatoid arthritis and neurological disorders. Until now, no (patho)physiological function has been ascribed to circulating Ig light chains. Here we show that IgLCs can confer mast cell-dependent hypersensitivity in mice. Antigenic stimulation results in plasma extravasation, cutaneous swelling and mast-cell degranulation. We show that IgLCs have a crucial role in development of contact sensitivity, which could be completely prevented by a novel IgLC antagonist. Although IgE and IgG₁ are central to the induction of immediate hypersensitivity reactions, our results show that IgLCs have similar activity. IgLCs may therefore be a novel factor in the humoral immune response to antigen exposure. Our findings open new avenues in investigating the pathogenesis of autoimmune diseases and their treatments.

Immunoglobulins (Ig) are important effector molecules of adaptive humoral immune responses. Production of antibodies to innocuous antigens is a reflection of a normal immune response. In allergic syndromes, IgE is thought to have a central role in eliciting immediate hypersensitivity reactions¹. Binding of IgE to its receptors, FcεRI, on mast cells and basophils and subsequent crosslinking of receptor-bound IgE triggers immediate hypersensitivity reactions. On the other hand, there is increasing evidence that hypersensitivity diseases do not always correspond with increased serum IgE levels. Studies in IgE-deficient mice suggest that active anaphylaxis can be induced by IgG₁ triggering the receptor (refs. 2,3) FcγRIII. Alternatively, similar hypersensitivity reactions can be elicited by other antigen-specific factors such as lymphocyte-derived picryl chloride factor⁴.

Tetrameric Ig are produced and secreted by plasma cells. However, it is well documented that plasma cells also produce and secrete Ig-free light chain (IgLC) in excess over Ig heavy chains^{5–8}. Turnover studies in humans demonstrated that only 60% of synthesized IgLC is incorporated into isotypic whole Ig and that the remaining fraction is released into serum as IgLCs (ref. 9). IgLCs are present in normal serum and can further be detected in urine and cerebrospinal fluid. Single IgLCs and IgLC dimers show specific binding to antigen. Affinities of IgLCs are generally lower than those of the parent tetrameric Igs, but comparable or higher affinities for light chains have been reported^{10–16}.

Currently, no (patho)physiological role for secreted IgLCs in immune responses has been documented, although they may be

involved in the degradation of neuropeptides^{17,18}. In this study, we show that IgLCs can transfer immediate hypersensitivity-like responses in mice. Exposure of IgLC-sensitized mice to antigen results in mast cell-dependent plasma extravasation and local tissue responses. We propose that IgLCs may contribute to the mechanism by which mast cells regulate immune-diseases.

IgLC transfers hapten-sensitivity

To obtain hapten-specific IgLCs, mouse trinitrophenol (TNP)-specific IgG₁ or oxazolone-specific IgG₁ was reduced and alkylated to separate light and heavy chains. Pure IgLCs and heavy chain fractions were isolated by gel filtration. Mice received purified TNP-specific IgLCs or heavy chains intravenously (i.v.) to test their ability to transfer hapten sensitivity. Two hours after topical challenge with hapten in IgLC recipients, an increase in ear thickness was measured. Significant increases in ear thickness could be detected as early as 30 min after hapten exposure. In contrast, mice receiving TNP-specific Ig heavy chain or phosphate buffered saline (PBS) did not elicit ear-tissue swelling after hapten challenge (Fig. 1a). Transfer of 0.5 μg IgLC per mouse resulted in significant ear swelling after hapten challenge, whereas maximal responses were found after injection of 2–5 μg IgLC (Fig. 1b). Subsequently, we studied the duration of hapten sensitivity after passive transfer of IgLC. The hapten-induced ear swelling remained constant up to 48 hours after IgLC transfer. Even 168 hours after transfer, a significant swelling was elicited (Fig. 1c). This time-course for IgLC transfer was similar to that found for passive transfer of monoclonal IgE (Fig. 1c). Local in-



jection of IgLC followed by systemic antigen challenge elicited passive cutaneous anaphylaxis (PCA), resulting in significant plasma extravasation in the dorsal skin (Fig. 1*d*). In the ear, PCA mediated by IgLC resulted in a rapid ear swelling after i.v. challenge (Fig. 1*e*). The responses in IgLC recipients are hapten-specific. In TNP- or oxazolone-specific IgLC recipients, ear swelling was only induced after challenge with the proper hapten (Fig. 2*a* and *b*).

We excluded the possibility that contamination with IgG₁ induced the cutaneous response. No ear swelling was detected after topical challenge when 100 ng, 500 ng or even 5 µg IgG₁ was used for passive systemic sensitization (data not shown). Moreover, in experiments using recombinant IgLC (by definition devoid of any heavy chains), we were able to passively transfer hapten sensitivity (Fig. 2*c*). Although both heavy and light chains contribute to antigen recognition in tetrameric Igs, our experiments demonstrate that IgLC alone is sufficient to confer hypersensitivity in naive recipients.

Host Ig is not involved in IgLC-elicited hypersensitivity

Free heavy chains are generally not secreted by B lymphocytes, and exchange of light chains within complete Igs has not been described. Hypothetically, exchange with IgE or IgG₁ or free heavy chains may have been responsible for the IgLC-induced effects. To investigate this possibility, we passively sensitized B cell-deficient mice (µMT), which lack circulating Igs (ref. 19). B cell-deficient mice were sensitized with TNP-specific IgLC, resulting in a cutaneous swelling after topical challenge (Fig. 2*d*). Compared with congenic littermates (C57BL/6), the degree of ear swelling in µMT mice was reduced. A similar reduction was

found in IgE-sensitized mice. This decreased response in the µMT mice for both IgE and IgLC may be due to decreased mast-cell survival in the absence of IgE or altered receptor expression^{20–23}. These experiments indicate that passive sensitization by IgLC does not depend on the presence of tetrameric Ig nor is it due to exchange with resident light chains.

Role of mast cells in IgLC responses

We next investigated the involvement of mast cells in IgLC-elicited responses. Passive sensitization of mast cell-deficient (W/W^v) mice with IgLC followed by topical application of hapten did not result in significant ear swelling. Control mast cell-sufficient littermates showed normal ear swelling responses (Fig. 3*a*). Re-establishment of the mast-cell population by local injection of bone marrow-derived mast cells (BMMC) restored the ear-swelling responses (W/W^v + BMMC) (Fig. 3*a*). Hapten challenge of IgLC-sensitized BALB/c mice was accompanied by rapid increase in plasma histamine levels *in vivo* (PBS group: 107 ± 16.6 nM versus TNP-specific IgLC 282 ± 95.7 nM, measured 5 min after i.v. challenge with TNP-conjugated albumin). Direct proof of mast-cell activation was obtained after histological and ultrastructural analysis of biopsies of the ears following topical hapten challenge. Mast cells showed marked signs of degranulation (Fig. 3*b*). Electron microscopy revealed that degranulation *in vivo* was characterized by swelling of intracytoplasmic granules, decrease of electron-density of the granules and extrusion of membrane-free granules from the mast cells (Fig. 3*c*). To further prove that IgLC directly mediated mast-cell activation, we sensitized BMMC with IgLC. IgE was used as a positive control. Subsequently, these cells were intradermally injected into W/W^v

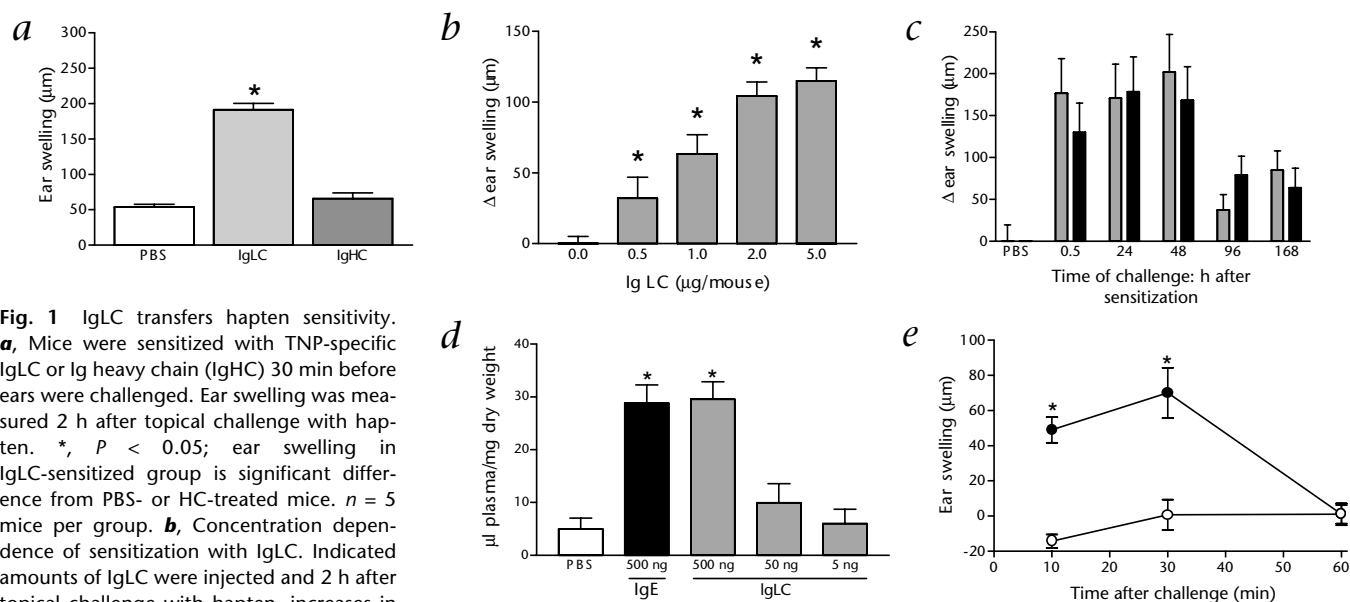


Fig. 1 IgLC transfers hapten sensitivity. **a**, Mice were sensitized with TNP-specific IgLC or Ig heavy chain (IgHC) 30 min before ears were challenged. Ear swelling was measured 2 h after topical challenge with hapten. *, $P < 0.05$; ear swelling in IgLC-sensitized group is significant difference from PBS- or HC-treated mice. $n = 5$ mice per group. **b**, Concentration dependence of sensitization with IgLC. Indicated amounts of IgLC were injected and 2 h after topical challenge with hapten, increases in ear thickness were measured and corrected for swelling induced by hapten only in PBS-injected group. *, $P < 0.05$; significant difference from non-sensitized group. $n = 4$ mice per group. **c**, Long-lasting sensitization with IgLC. Mice were intravenously sensitized with TNP-specific IgLC (■) or IgE (●) (5 µg per mouse). At different time points after sensitization, mice were topically challenged with hapten and increase in ear thickness was measured at 2 h after challenge. Data are corrected for swelling induced by hapten only in the non-sensitized (PBS) group. At all time points, IgLC- or IgE-treated groups showed significant ear swelling. $P < 0.05$; $n = 4$ –6 mice per group. **d**, Topical challenge leads to plasma extravasation in IgLC-sensitized mice. Mice were injected intra-

dermally with indicated amounts of TNP-specific IgLC, TNP-specific IgE or PBS and subsequently challenged with hapten. Induction of vascular leakage was assessed 2 h later by quantification of accumulated Evans blue. *, $P < 0.05$, significant difference from PBS-treated group; $n = 4$ mice per group. **e**, Passive cutaneous anaphylaxis reaction induced by IgLC. Ears of mice were sensitized by intradermal injection with IgLC (●) or vehicle (○). 20 h after sensitization, all mice received an i.v. injection with hapten-conjugated albumin and ear swelling was monitored at different time points after challenge. *, $P < 0.05$; significant difference from PBS-treated group; $n = 4$ mice per group. Error bars represent s.e.m.

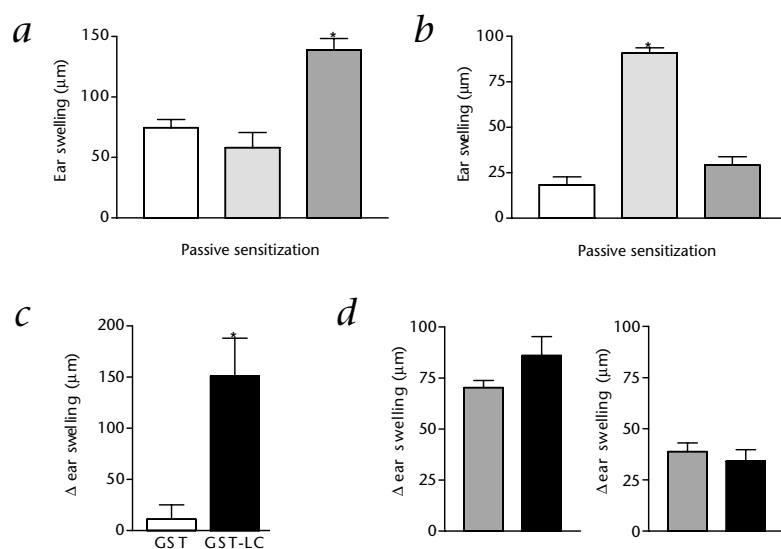


Fig. 2 Antigen-specificity of sensitization with IgLC. **a** and **b**, Mice were injected with oxazolone-specific IgLC (■), TNP-specific IgLC (■) or PBS (□). 30 min after injection, ears were challenged with picryl chloride (**a**) or oxazolone (**b**). Ear swelling was measured at 2 h after challenge. *, $P < 0.05$, significant difference from PBS-treated group; $n = 6$ mice per group. **c**, Mice were passively sensitized with recombinant GST-IgLC (■) or recombinant GST only (□). Ear swelling was measured 2 h after hapten challenge. Data expressed are corrected for swelling induced by application of hapten only in the PBS group. $P < 0.05$, $n = 5$ mice per group. **d**, Hapten-induced ear swelling in Ig LC-sensitized B cell-deficient mice. B cell-deficient (μMT) (right) or congenic controls (C57BL/6) (left) were sensitized with TNP-specific IgLC (■) or TNP-specific IgE (■). Ear swelling was monitored at 2 h after hapten challenge. Data are corrected for swelling induced by application of hapten only in the non-sensitized (PBS) group. Ear swelling in μMT was significantly different from C57BL/6 for both experimental groups, $P < 0.05$. No significant difference was found between IgE- and IgLC-sensitized groups. $n = 5$ mice per group. Error bars represent s.e.m.

mice. Systemic challenge with hapten-conjugated albumin resulted in increased plasma leakage at the site of injection of IgLC- and IgE-sensitized mast cells as compared with the site of injection of non-sensitized mast cells or PBS (Fig. 3d). Injection of IgLC or IgE only (without mast cells) did not induce leakage after hapten challenge (data not shown). Moreover, primary cultured mast cells could be activated after crosslinking of cell-surface proteins with IgLC, which resulted in release of granule constituents (β-hexosaminidase) (Fig. 3e) and production of leukotrienes (Fig. 3f). In agreement with ear-swelling experiments, Ig heavy chain did not induce release of mast-cell mediators. This indicates that IgLC can directly mediate mast-cell activation and mediator release by crosslinking of mast-cell surface proteins.

IgLC does not activate γ-chain-associated Fc receptors

In our previous experiments, we demonstrated that mast cells are essential for the IgLC-induced cutaneous responses. Furthermore, mast cells could be activated after crosslinking of cell-surface proteins with IgLC. Next, we investigated whether direct binding of IgLC to mast cells could be detected and which proteins are involved in binding. BMMCs were incubated with fluorescein isothiocyanate (FITC)-labeled IgLC or IgE, and binding was monitored using fluorescence microscopy. IgLC binds to mast cells (Fig. 4b) and fluorescence intensity suggests a patchy distribution of IgLC-binding proteins (receptors) on the mast-cell membrane. Such clustered distribution has also been demonstrated earlier for FcεRI (ref. 24) (Fig. 4c). In a second set

of experiments, we used beads derivatized with IgLC or IgE to visualize binding to mast cells. IgLC- and IgE-derivatized beads formed rosettes around mast cells, whereas albumin-coated beads did not (Fig. 4d–f). The beads were then used to isolate membrane proteins involved in IgLC binding (Fig. 4g). Using IgLC-derivatized magnetic beads, we were able to purify a mast cell-derived membrane protein with an apparent molecular weight of 45 kD (Fig. 4g). Ongoing research is focused on the identification of this protein.

In another set of experiments, we tested whether IgLC bound to mast cells was able to bind hapten. BMMCs preincubated with TNP-specific IgLC were combined with hapten-derivatized sheep red blood cells and rosetting was scored by light microscopy. We show that TNP-labeled cells bind IgLC-sensitized mast cells but unlabeled or oxazolone-labeled cells do not (Fig. 4h), demonstrating that surface-bound IgLC recognizes antigen.

Crosslinking of FcγRIII or FcεRI receptors is well known to activate mast cells in an antigen-specific manner. Both receptors signal via the common γ chain and can trigger hypersensitivity reactions. Passive sensitization with IgLC of common γ chain-deficient (FcγR^{-/-}) mice resulted in similar ear-swelling responses after hapten challenge as compared with wild-type (C57BL/6) mice (Fig. 4i). As expected, the response to IgE was completely abolished in the knockout mice. This indicates that IgLC interacts with a receptor that does not need the common γ chain for signaling and thereby excludes FcεRI and FcγRIII as effector molecules in IgLC-induced hypersensitivity reactions. These results are in accordance with the findings in the B cell-deficient mice demonstrating that no exchange with IgE or IgG₁ was necessary for the effects induced by IgLC (Fig. 2c).

IgLCs are produced upon contact sensitization of mice

We examined if antigen exposure of naive mice could elicit IgLC production. To test this, mice were sensitized epicutaneously with picryl chloride, dinitrofluorobenzene (DNFB) or oxazolone and 4 days after sensitization, spleen and draining lymph node cells were cultured *in vitro* for 24 hours in the absence of hapten. Hapten-specific proteins from culture supernatant were isolated by hapten-affinity chromatography²⁵. TNP-specific proteins were isolated from the culture supernatant of spleen and lymph-node cells isolated from mice as early as one day after topical sensitization with picryl chloride (TNP). No such proteins were isolated from naive mice. Similar to purified IgLC, we could passively sensitize naive mice with these hapten-specific factors.

The bioactivity of the TNP-specific factor was not affected by either pretreatment with protein A/G-beads or reduction plus alkylation, and it was heat labile (data not shown). Moreover, presence of Ig-κ light chain is essential for the bioactivity. Pretreatment with immobilized antibody specific for Ig-κ light chain, but not with an irrelevant antibody (murine Vβ8-specific), completely abolished the capacity of this antigen-binding protein to transfer hapten sensitivity to naive mice (Fig. 5a). Collectively, these results indicate that intact Ig was not responsible for the bioactivity of these factors⁴. In contrast to human light chains, no reagents are available to distinguish between

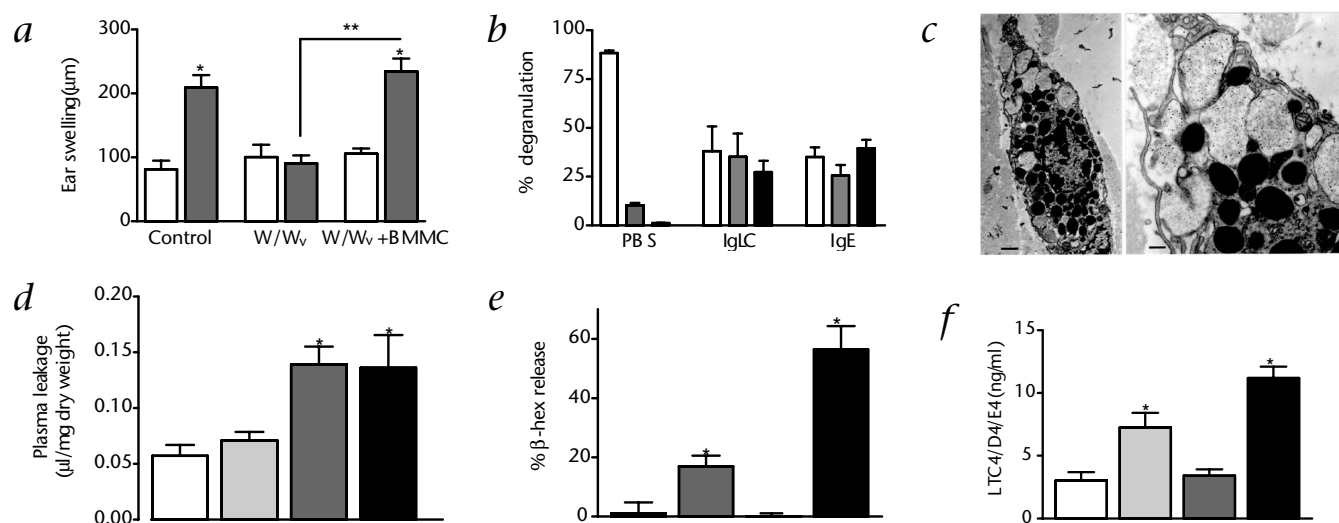


Fig. 3 Mast-cell dependency of hapten-induced ear swelling in IgLC-sensitized mice. **a**, Mast cell-deficient (*W/W^v*), mast cell-reconstituted (*W/W^v*+BMMC) and congenic controls were sensitized with TNP-specific IgLC (■) or vehicle (□). 30 min after sensitization mice were topically challenged with picryl chloride and ear swelling was determined after 2 h. $n = 6$ mice per group. **, $P < 0.05$, Ear swelling in *W/W^v* was significantly different from control mice. No differences were found between control and *W/W^v*-reconstituted mice. *Significantly different from PBS, $P < 0.05$. **b**, Distribution of mast cell populations at the site of ear skin reactions according to the degree of degranulation. Cells showed no degranulation (□), mild (■) or extensive (■) degranulation based on staining of granules. <50% of granules in mildly degranulated cells and most granules in extensively degranulated cells stained lightly or metachromatically. Groups of mice ($n = 3$) received TNP-specific IgLC (5 μ g), TNP-specific IgE (5 μ g) or PBS. All mice were challenged by topical application of the hapten to the ears. Degranulation was scored at 1 h after topical challenge. Mean $\pm$ s.d. was calculated for each group of mice. TNP-specific IgLC and IgE recipients showed a significant rise in mild and extensive degranulation, $P < 0.05$. 2–5 tissue specimens from 3 different mice were analyzed. **c**, Electron micrograph of a mildly degranulated mast cell in the dermis of the ear of an IgLC-sensitized mouse at 1 h after topical challenge with hapten. Right panel shows enlargement. Scale bars, 1,100 nm (left); 350 nm (right). **d**, Vascular leakage induced by activation of *in vitro*-sensitized mast cells. BMMC were sensitized with TNP-specific IgLC or TNP-specific IgE. Subsequently, PBS (□) untreated BMMCs (■), IgLC-sensitized BMMCs (■) or IgE-sensitized BMMCs (■) were injected in the basolateral skin from *W/W^v* mice. Vascular leakage at the site of injection was assessed by accumulation of Evans blue at 2 h after systemic challenge. $n = 6$ mice per group. *, $P < 0.05$, difference from untreated BMMC or PBS. **e** and **f**, *In vitro* activation of mast cells after crosslinking of cell surface proteins with IgLC. Primary cultured pulmonary mast cells were incubated with beads, which were either non-derivatized (control; □) or derivatized with IgLC (■), Ig heavy chain (IgHC; ■) or IgE (■). After incubation, supernatant was collected to assess release of β -hexosaminidase (**e**) or leukotriene levels (**f**). Values represent mean $\pm$ s.e.m., $n = 4$. * $P < 0.05$, difference from control-beads. Error bars represent s.e.m.

free and bound Ig light chains of mice. Therefore, we analyzed the hapten-specific proteins by immunoblotting with Ig light and heavy chain-specific antibodies. Western-blot analysis showed that the isolated hapten-specific protein factors indeed contained Ig (κ) light chains (Fig. 5b), whereas no Ig heavy chains from IgG and IgM (Fig. 5c, lane 6) or IgE (Fig. 5d, lane 6) were detected. The identity of this protein was further confirmed by sequencing the N-terminal amino acids from a purified protein of 27 kD, which transferred hapten sensitivity. The sequence showed complete homology to mouse Ig- κ light chain. Moreover, in spleen-cell culture supernatants from picryl chloride-sensitized B cell-deficient mice (μ MT) and Ig- κ light chain (CkT)-knockout mice, no immunoreactive Ig- κ light chain was detected. This confirms the presence of Ig- κ light chains in hapten-specific factors and indicates the involvement of a B-lymphocyte population in production of antigen-specific IgLC.

F991 is an Ig free light chain antagonist

Tamm-Horsfall protein (THP), a monomeric glycoprotein produced by cells in the ascending limb of Henle of the kidney, has a specific binding affinity to IgLC. F991 is a 9-mer peptide derived from the amino-acid sequence in THP responsible for binding²⁶. We tested the ability of this compound to inhibit sensitization with IgLC. Mice were passively sensitized with IgLC and subsequently challenged with hapten on the ear to induce ear swelling. I.v. injection of F991 dose-dependently inhibited

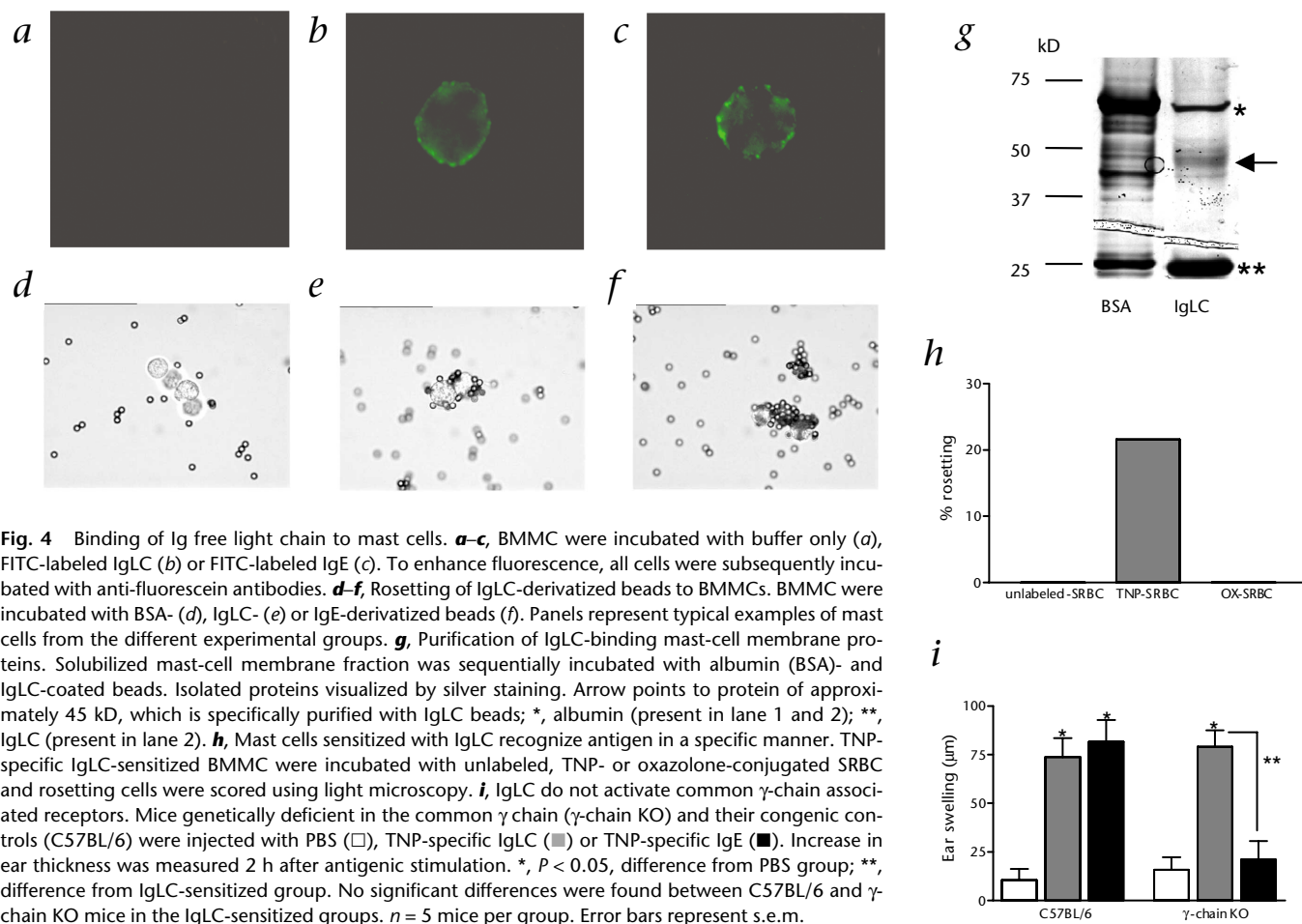
IgLC-mediated ear swelling after hapten challenge (Fig. 6a). F991 appears specific for IgLC, because cutaneous anaphylaxis induced by IgE or IgG₁ was not affected by pretreatment with this compound (Fig. 6b). When contact-sensitized mice are challenged on the ear, a biphasic ear-swelling response is elicited. An early swelling is notable after 30 minutes for up to 2 hours after challenge, and is followed by a late phase swelling between 24 and 48 hours²⁷. If IgLC has an important role in evoking contact sensitivity, the prediction is that F991 would be able to inhibit this. Indeed, F991 completely inhibited both early and delayed ear swelling, indicating that IgLC mediates a crucial step in the immune response to contact allergens in mice (Fig. 6c and d).

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Discussion

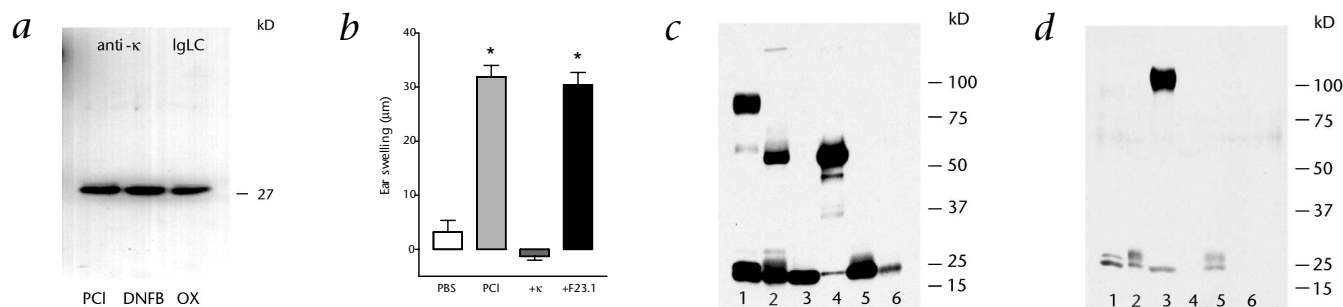
Here we provide evidence for a novel role for IgLC in adaptive immune responses. So far, a functional role for circulating IgLC in immune responses has not been documented. Our results show that IgLC can transfer hapten sensitivity and subsequent antigenic challenge triggers responses reminiscent of immediate hypersensitivity reactions induced by IgE. In mast cell-deficient mice, an IgLC-induced cutaneous reaction could not be elicited, whereas reconstitution of the mast-cell population reestablished this response. Mast-cell activation was accompanied by morphological changes and release of mast-cell mediators in serum. Intradermal injection of IgLC or IgLC-sensitized mast cells fol-



lowed by antigen challenge resulted in a PCA reaction associated with tissue swelling and plasma extravasation. *In vitro* crosslinking of mast-cell surface proteins with IgLC induced degranulation and lipid-mediator production (Fig. 3e and f).

Several lines of evidence suggest the presence of a receptor for IgLC on mast cells. First, passive transfer of IgLC resulted in long-lasting sensitization, whereas circulating IgLC has only a short half-life, suggesting that IgLC is bound to a target molecule

to induce sensitization. Second, binding experiments show direct interaction of IgLC with mast cells, and membrane proteins specifically bound to IgLC were isolated. Finally, crosslinking of mast-cell surface proteins with IgLC results in mast-cell activation. We have not yet identified the putative IgLC. We show in knockout mice that γ -chain associated receptors such as Fc ϵ RI, Fc γ RIII (ref. 28), and PIR-A (ref. 29) are not involved in IgLC-triggered mast-cell activation. Moreover, IgLC-mediated mast-cell



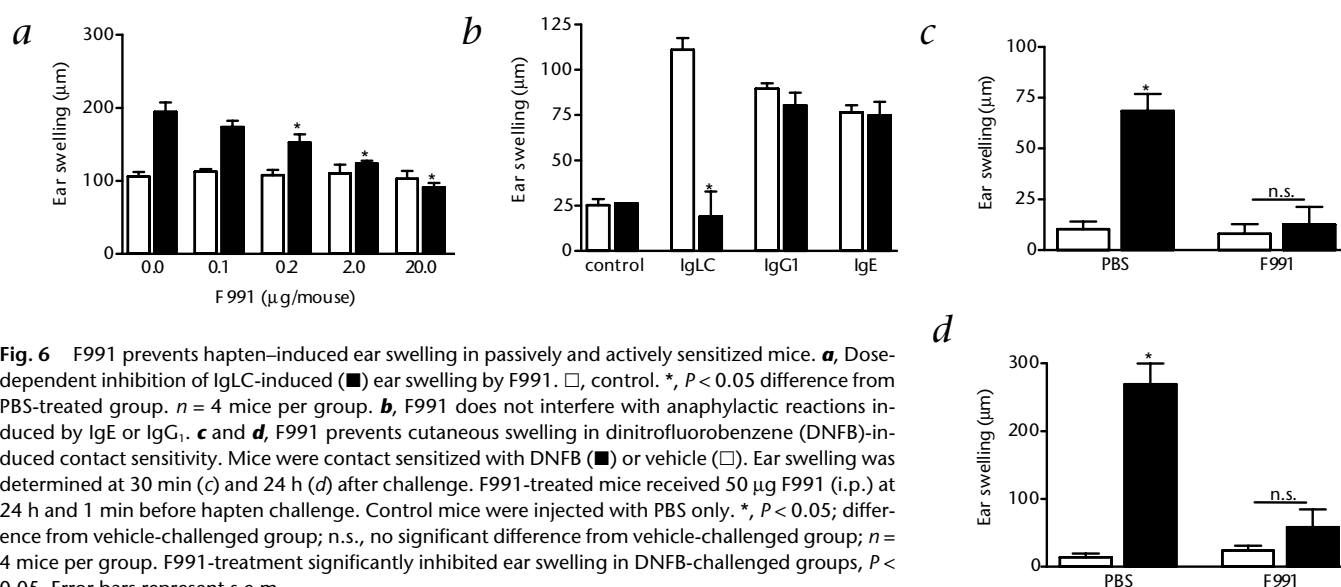


Fig. 6 F991 prevents hapten-induced ear swelling in passively and actively sensitized mice. **a**, Dose-dependent inhibition of IgLC-induced (■) ear swelling by F991. □, control. *, $P < 0.05$ difference from PBS-treated group. $n = 4$ mice per group. **b**, F991 does not interfere with anaphylactic reactions induced by IgE or IgG₁. **c** and **d**, F991 prevents cutaneous swelling in dinitrofluorobenzene (DNFB)-induced contact sensitivity. Mice were contact sensitized with DNFB (■) or vehicle (□). Ear swelling was determined at 30 min (c) and 24 h (d) after challenge. F991-treated mice received 50 μ g F991 (i.p.) at 24 h and 1 min before hapten challenge. Control mice were injected with PBS only. *, $P < 0.05$; difference from vehicle-challenged group; n.s., no significant difference from vehicle-challenged group; $n = 4$ mice per group. F991-treatment significantly inhibited ear swelling in DNFB-challenged groups, $P < 0.05$. Error bars represent s.e.m.

activation is independent of complement activation (data not shown). The number of receptors implicated in activation of mast cells and other immune cells is increasing^{30–32}, and for many of these receptors ligands are still unknown. We have isolated a mast-cell membrane associated protein that interacts with IgLC and are in the process of determining its role in IgLC-mediated hypersensitivity-like reactions.

Mast cells have been implicated in a wide variety of biological responses including immediate hypersensitivity reactions, bacterial sepsis³³ and in T cell-dependent reactions such as contact sensitivity³⁴, experimental autoimmune encephalomyelitis (EAE)³⁵, rheumatoid arthritis³⁶ or non-atopic asthma (data not shown). Diseases like contact sensitivity, EAE, rheumatoid arthritis and non-atopic asthma can be induced by local delayed-type hypersensitivity (DTH) reactions, which are thought to be independent of IgE or IgG₁. Several studies in mast cell-deficient animals show that mast cells are important amplifiers of DTH reactions^{34,35,37}. Vasoactive mediators released by mast cells have a role in initiation and amplification of DTH responses by increasing permeability of the vasculature and orchestrating attraction of infiltrating cells³⁴. Our experiments suggest that mast cells can be activated during a DTH reaction by the crosslinking of surface-bound IgLC. Notably, antigen-specific humoral factors released by B cells are crucial in contact hypersensitivity reactions, as hypersensitivity is impaired in B-cell- and B-1-deficient animals²⁷. IgLC can rescue this impaired contact hypersensitivity reaction in pan B-cell and B-1 deficient mice (data not shown). Here we show that contact sensitization with different haptens results in the production of IgLC by spleen and lymph-node cells. Contact sensitization may rapidly activate B cells, likely B-1 cells, to release antibodies and antibody fragments, independent of T cells³⁸. However, an involvement of B-2 cells at later stages after sensitization cannot be excluded^{5–8}. The role of IgLC in immune responses to more complex antigens needs to be further examined.

Our results show that the IgLC antagonist F991 inhibits the development of early and delayed ear swelling in DNFB-induced contact sensitivity, which indicates a crucial role for IgLC in these reactions. In humans, the secretion of light chains is augmented under pathological conditions such as multiple sclerosis,

rheumatoid arthritis, Sjögren disease, systemic lupus erythematosus, and other neurological disorders^{39–41}. For example, production of IgLC in patients with multiple sclerosis and rheumatoid arthritis is greatly enhanced and correlates with severity of the disease⁴¹. Furthermore, there is substantial evidence describing the involvement of mast cells in the pathogenesis of these IgLC-associated diseases^{35,42,43}. In preliminary experiments, treatment with F991 prevented development of clinical signs of disease in an animal model for experimental allergic encephalomyelitis. Further, we have found that passive sensitization with IgLC gives rise to a rapid and profound airway bronchoconstriction in mice after intra-airway challenge, a response dependent on mast cell activation (data not shown). This suggests that IgLC may play a role in non-atopic asthmatic responses and predicts a therapeutic effect of IgLC antagonists. Indeed the development of airway hyperreactivity and inflammation in a murine model for non-atopic asthma could be inhibited by F991 (data not shown).

In conclusion, our study introduces a new concept in which IgLC can elicit immediate hypersensitivity-like responses. The production of IgLC may provide a functional link between the mast cell and the development of non-allergic responses and suggests new avenues to investigate the pathogenesis and treatment autoimmune diseases.

Methods

Isolation of hapten-specific IgLC. TNP-specific (1B7-11, ATCC) (American Type Culture Collection, Manassas, Virginia) or oxazolone-specific IgG (NQ10/12.5) (provided by C. Milstein) were purified with protein G-sepharose (Amersham Biosciences, Roosendaal, the Netherlands). IgG was reduced and alkylated. Heavy and light chains were purified by gel filtration, essentially as described¹² and dialyzed against PBS. IgLC was absorbed with protein-G sepharose. Purity was verified by SDS-PAGE and protein staining. Recombinant IgLC was produced by PCR cloning of the cDNA fragment encoding V_L-C_L from 1B7-11 in a pGEX vector (Amersham Biosciences, Roosendaal, the Netherlands). Fusion proteins were expressed in *Escherichia coli* and purified using affinity chromatography.

Measurement of ear swelling. BALB/c (GDL, Utrecht, the Netherlands), μ MT and C57BL/6, mast cell-deficient mice (WBB6F₁-W/W⁺) and normal littermates (WBB6F₁-+/+) (The Jackson Laboratory, Bar Harbor, Maine) or FcR γ ^{-/-} mice were injected i.v. with the indicated concentrations of TNP-spe-



cific IgLC or TNP-specific IgE (2–5 µg/mouse). 30 min after injection, ears were topically challenged with 0.8% PCI (picryl chloride) in acetone/olive oil or with 0.8% oxazolone as indicated. 2 h after challenge, ear thickness was determined with an engineer's micrometer. Mast-cell reconstitution was performed by injection of 0.5×10^6 bone marrow-derived mast cells (BMMC) into both ears of W/W^m mice at 6 wk before passive sensitization. BMMC were cultured from bone marrow of WBB6F₁-/+ mice⁴⁴.

Passive cutaneous anaphylaxis. Right ears of BALB/c mice were injected intradermally with 500 ng TNP-specific IgLC and left ears with PBS. Mice received 100 µg DNP-conjugated human serum albumin (DNP-HSA) (i.v.) 20 h later.

Measurement of plasma extravasation. Mice were injected intradermally at the basolateral site with IgLC, IgE or PBS alone. 20 min later, mice received 100 µg DNP-HSA i.v. mixed with 1.25% Evans blue. Tissue from the injection spots was excised 2 h later. Evans blue was extracted in formamide (18 h at 40 °C) and quantified at 595 nm. Vascular leakage was calculated as the ratio of Evans blue in tissue and plasma.

Plasma extravasation induced by BMMC. BMMC from BALB/c were sensitized with TNP-specific IgLC or TNP-specific IgE. 5×10^4 BMMC were injected at the basolateral site of W/W^m mice. Mice were challenged and leakage was assessed as described above.

Antagonist studies. F991 (AHWSGHCCCL) was synthesized by Fmoc chemistry (Ansynth, Roosendaal, the Netherlands). In passive transfer studies with TNP-specific IgLC (2 µg), IgE (2 µg), IgG₁ (100 µg) or F991 (50 µg, intraperitoneally (i.p.)) was administered 3.5 h before sensitization. The right ear was painted with PCI and the left ear with vehicle 30 min after transfer. Ear thickness was measured 2 h after challenge. DNFB sensitization and challenge were performed as described²⁵. In actively sensitized mice, F991 (50 µg, i.p.) was administered 24 h and 1 min before hapten challenge.

Electron microscopy and histology. BALB/c mice sensitized with TNP-specific IgLC or IgE. Hapten was applied on the ears 30 min later. Ear samples were fixed in Karnovsky 1 h after challenge. Samples were cut and washed with osmium tetroxide, dehydrated and embedded on epoxy resin. Semi-thin sections were stained with toluidine blue. Mast cells were examined in 10 sections per mouse. Ultrathin sections were contrasted with aqueous uranyl acetate and Reynolds lead citrate and examined in a Philips 201 TEM (Philips Industries, Eindhoven, the Netherlands).

Isolation and analysis of hapten-binding factor. BALB/c were skin-sensitized with recrystallized picryl chloride, DNFB or oxazolone as described²⁵. 4 d after sensitization, spleen and lymph node cells (10^7 cells per ml) were cultured for 24–48 h in RPMI. The supernatant was loaded on to a hapten-affinity column and proteins were eluted with guanidine. Samples were dialyzed versus PBS and tested (70–80 µg) for bioactivity in an ear-swelling test. TNP-binding protein was pretreated for 1 hour at 4 °C with Ig-κ LC-specific antibody (The Binding Site, Birmingham, UK) or irrelevant antibody (F23.1 monoclonal antibody) immobilized on sepharose beads. Unbound proteins were tested for bioactivity.

Hapten-binding proteins (4.5 µg) were fractionated under reducing conditions and immunoblotted with Ig-κ light chain-specific antibody (The Binding Site), mouse IgE-specific antibody (Pharmingen, Woerden, the Netherlands) or mouse IgG-, IgA-, IgM-specific antibody (ICN, Zoetermeer, the Netherlands). Purified mouse IgM (3 µg), IgG (3 µg), IgE (3 µg), heavy chain (γ₁) (1.5 µg), and IgLC (κ) (1.5 µg) were coelectrophoresed under reducing conditions. Immunoreactive proteins were visualized with ECL (Amersham, Roosendaal, the Netherlands).

Rosetting assays. Dynabeads M-280 Tosyl-activated (DynaL, Oslo, Norway) were coated with IgLC plus BSA or BSA alone according to the manufacturer's protocol. Dynabeads and BMMC were washed with Tyrode containing 10 mM HEPES and 0.1 % BSA (pH 7.2). BMMCs were incubated with Dynabeads on ice. After collection, beads were washed and rosetting was monitored using light microscopy. BMMC were incubated with TNP-specific IgLC at 37 °C for 60 min, washed and BMMC were mixed with 0.5% haptenized sheep red blood cells (SRBC). After 1 h incubation at room tempera-

ture, cells were pelleted and resuspended in 0.2% glutaraldehyde/PBS. Percentage of rosetting cells (>4 SRBC per cell) was scored using light microscopy.

Fluorescence microscopy. BMMC were resuspended in PBS supplemented with FCS/azide and incubated for 45 min on ice with 1 µg IgLC-FITC, IgE-FITC or buffer only. After washing, cells were incubated with 1 µg AlexaFluor488-conjugated anti-FITC (Molecular Probes, Leiden, the Netherlands), washed and examined by fluorescence microscopy.

Isolation of membrane proteins. BMMC were lysed and centrifuged to remove cytoplasmic proteins. The pellet was resuspended and incubated for 30 min on ice in 50 mM Tris, 150 mM NaCl, 0.5 % NP-40, and protease inhibitors (pH 7.4). The lysate was centrifuged for 30 min at 14,000g. The supernatant was precleared with BSA-coated Dynabeads and then incubated with IgLC-coated Dynabeads for 30 min at 4 °C. After washing, proteins were eluted from beads, separated by SDS-PAGE followed by silver staining.

In vitro activation of mast cells. Pulmonary mast cells were cultured as described⁴⁵. Mast cells were resuspended in Tyrode's buffer/10 mM HEPES. Subsequently, dynabeads were added and the mixture was incubated for 30 min at 37 °C. Cell-free supernatant was collected for measurement of β-hexosaminidase activity, as described⁴⁴. Leukotrienes (LT) were measured with an immunoassay (Amersham, Roosendaal, the Netherlands).

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Competing interests statement

The authors declare competing financial interests: see the website (<http://medicine.nature.com>) for details.

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1. Corry, D.B. & Kheradmand, F. Induction and regulation of the IgE response. *Nature* **402**, 818–823 (1999).
2. Oettgen, H.C. *et al.* Active anaphylaxis in IgE-deficient mice. *Nature* **370**, 367–370 (1994).
3. Miyajima, I. *et al.* Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc γRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.* **99**, 901–914 (1997).
4. Van Loveren, H. *et al.* Immune serum from mice contact-sensitized with picryl chloride contains an antigen-specific T cell factor that transfers immediate cutaneous reactivity. *Eur. J. Immunol.* **16**, 1203–1208 (1986).
5. Hopper, J.E. & Papagiannis, E. Evidence by radioimmunoassay that mitogen-activated human blood mononuclear cells secrete significant amounts of light chain Ig unassociated with heavy chain. *Cell. Immunol.* **101**, 122–131 (1986).
6. Shapiro, A.L., Scharff, M.D., Maizel, J.V. & Uhr, J.W. Synthesis of excess light chains of γ globulin by rabbit lymph node cells. *Nature* **211**, 243–245 (1966).
7. Skvortsov, V.T. & Gurvich, A.E. Relative rates of synthesis of immunoglobulins and light chains in rabbit spleen cells during secondary response. *Nature* **218**, 377–378 (1968).
8. Hannam-Harris, A.C. & Smith, J.L. Free immunoglobulin light chain synthesis by human foetal liver and cord blood lymphocytes. *Immunology* **43**, 417–423 (1981).
9. Waldmann, T.A., Strober, W. & Mogielnicki, R.P. The renal handling of low molecular weight proteins. II. Disorders of serum protein catabolism in patients with tubular proteinuria, the nephrotic syndrome, or uremia. *J. Clin. Invest.* **51**, 2162–2174 (1972).
10. Yoo, T.J., Roholt, O.A. & Pressman, D. Specific binding activity of isolated light chains of antibodies. *Science* **157**, 707–709 (1967).
11. Masat, L., Wabl, M. & Johnson, J.P. A simpler sort of antibody. *Proc. Natl. Acad. Sci.*

- USA **91**, 893–896 (1994).
12. Sun, M., Li, L., Gao, Q.S. & Paul, S. Antigen recognition by an antibody light chain. *J. Biol. Chem.* **269**, 734–738 (1994).
 13. Ledbetter, J.A., Fell, H.P., Grosmaire, L.S., Norris, N.A. & Tsu, T.T. An immunoglobulin light chain dimer with CD4 antigen specificity. *Mol. Immunol.* **24**, 1255–1261 (1987).
 14. Schechter, I. & Ziv, E. Binding of 2,4-dinitrophenyl derivatives by the light chain dimer obtained from immunoglobulin A produced by MOPC-315 mouse myeloma. *Biochemistry* **15**, 2785–2790 (1976).
 15. Painter, R.G., Sage, H.J. & Tanford, C. Contributions of heavy and light chains of rabbit immunoglobulin G to antibody activity. I. Binding studies on isolated heavy and light chains. *Biochemistry* **11**, 1327–1337 (1972).
 16. Tribbick, G., Edmundson, A.B., Mason, T.J. & Geysen, H.M. Similar binding properties of peptide ligands for a human immunoglobulin and its light chain dimer. *Mol. Immunol.* **26**, 625–635 (1989).
 17. Thiagarajan, P. *et al.* Monoclonal antibody light chain with prothrombinase activity. *Biochemistry* **39**, 6459–6465 (2000).
 18. Sun, M., Gao, Q.S., Li, L. & Paul, S. Proteolytic activity of an antibody light chain. *J. Immunol.* **153**, 5121–5126 (1994).
 19. Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* **350**, 423–426 (1991).
 20. Kalesnikoff, J. *et al.* Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* **14**, 801–811 (2001).
 21. Asai, K. *et al.* Regulation of mast cell survival by IgE. *Immunity* **14**, 791–800 (2001).
 22. Yamaguchi, M. *et al.* IgE enhances mouse mast cell Fc(epsilon)RI expression *in vitro* and *in vivo*: Evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* **185**, 663–672 (1997).
 23. Hsu, C. & MacGlashan, D., Jr. IgE antibody up-regulates high affinity IgE binding on murine bone marrow-derived mast cells. *Immunol. Lett.* **52**, 129–134 (1996).
 24. Sheets, E.D., Holowka, D. & Baird, B. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J. Cell. Biol.* **145**, 877–887 (1999).
 25. Meade, R., Van Loveren, H., Parmentier, H., Iverson, G.M. & Askenase, P.W. The antigen-binding T cell factor PCI-F sensitizes mast cells for *in vitro* release of serotonin. Comparison with monoclonal IgE antibody. *J. Immunol.* **141**, 2704–2713 (1988).
 26. Huang, Z.Q. & Sanders, P.W. Localization of a single binding site for immunoglobulin light chains on human Tamm-Horsfall glycoprotein. *J. Clin. Invest.* **99**, 732–736 (1997).
 27. Tsuji, R.F. *et al.* Required early complement activation in contact sensitivity with generation of local C5-dependent chemotactic activity, and late T cell interferon γ : A possible initiating role of B cells. *J. Exp. Med.* **186**, 1015–1026 (1997).
 28. Takai, T., Li, M., Sylvestre, D., Clynes, R. & Ravetch, J.V. FcR γ chain deletion results in pleiotropic effector cell defects. *Cell* **76**, 519–529 (1994).
 29. Maeda, A., Kurosaki, M. & Kurosaki, T. Paired immunoglobulin-like receptor (PIR)-A is involved in activating mast cells through its association with Fc receptor γ chain. *J. Exp. Med.* **188**, 991–995 (1998).
 30. Lee, K.H., Ono, M., Inui, M., Yuasa, T. & Takai, T. Stimulatory function of gp49A, a murine Ig-like receptor, in rat basophilic leukemia cells. *J. Immunol.* **165**, 4970–4977 (2000).
 31. Pirozzi, G., Terry, R.W., Epstein, D. & Labow, M.A. Identification and characterization of a novel surface antigen gene induced in mast cells activated through the high affinity IgE receptor. *J. Immunol.* **155**, 5811–5818 (1995).
 32. Smith, D.A., Monk, P.N. & Partridge, L.J. Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells. *Mol. Immunol.* **32**, 1339–1344 (1995).
 33. Echtenacher, B., Mannel, D.N. & Hultner, L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381**, 75–77 (1996).
 34. Biedermann, T. *et al.* Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *J. Exp. Med.* **192**, 1441–1452 (2000).
 35. Secor, V.H., Secor, W.E., Gutekunst, C.A. & Brown, M.A. Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J. Exp. Med.* **191**, 813–822 (2000).
 36. Woolley, D.E. & Tetlow, L.C. Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion. *Arthritis Res.* **2**, 65–74 (2000).
 37. Geba, G.P. *et al.* Delayed-type hypersensitivity in mast cell-deficient mice: Dependence on platelets for expression of contact sensitivity. *J. Immunol.* **157**, 557–565 (1996).
 38. Askenase, P.W. & Tsuji, R.F. B-1 B cell IgM antibody initiates T cell elicitation of contact sensitivity. *Curr. Top. Microbiol. Immunol.* **252**, 171–177 (2000).
 39. Fagnart, O.C., Sindic, C.J. & Laterre, C. Free κ and λ light chain levels in the cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases. *J. Neuroimmunol.* **19**, 119–132 (1988).
 40. Moutsopoulos, H.M., Steinberg, A.D., Fauci, A.S., Lane, H.C. & Papadopoulos, N.M. High incidence of free monoclonal λ light chains in the sera of patients with Sjogren's syndrome. *J. Immunol.* **130**, 2663–2665 (1983).
 41. Solling, K., Solling, J. & Romer, F.K. Free light chains of immunoglobulins in serum from patients with rheumatoid arthritis, sarcoidosis, chronic infections and pulmonary cancer. *Acta Med. Scand.* **209**, 473–477 (1981).
 42. Rozniecki, J.J., Hauser, S.L., Stein, M., Lincoln, R. & Theoharides, T.C. Elevated mast cell tryptase in cerebrospinal fluid of multiple sclerosis patients. *Ann. Neurol.* **37**, 63–66 (1995).
 43. Marone, G. Mast cells in rheumatic disorders: Mastermind or workhorse? *Clin. Exp. Rheumatol.* **16**, 245–249 (1998).
 44. Karimi, K., Redegeld, F.A., Heijdra, B. & Nijkamp, F.P. Stem cell factor and interleukin-4 induce murine bone marrow cells to develop into mast cells with connective tissue type characteristics *in vitro*. *Exp. Hematol.* **27**, 654–662 (1999).
 45. Lukacs, N.W. *et al.* The role of stem cell factor (c-kit ligand) and inflammatory cytokines in pulmonary mast cell activation. *Blood* **87**, 2262–2268 (1996).