

The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell activation by limiting B cell antigen receptor surface expression

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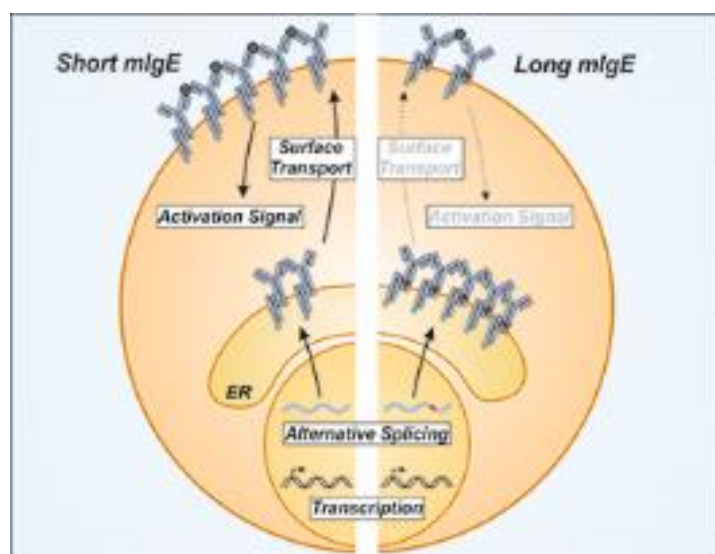
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Abbreviations: 7-AAD: 7-Aminoactinomycin D, Akt: RAC-alpha serine/threonine-protein kinase, BCR: B cell antigen receptor, AP: affinity purification, EMPD: extracellular membrane-proximal domain, ER: endoplasmic reticulum, Ig α/β : immunoglobulin α/β , Grb2: growth factor receptor-bound 2, Ig: immunoglobulin, ITAM: immunoreceptor tyrosine-based activation motif, mIgE: membrane-bound IgE, SLP65: SH2 domain-containing leukocytes adapter protein of 65 kDa, PLC- γ 2: phospholipase C- γ 2, PI3' kinase: phosphatidylinositol 3' kinase, PTK: protein tyrosine kinase, Erk: extracellular signal-regulated kinase, Syk: spleen tyrosine kinase

Abstract

Immunoglobulin E (IgE) antibodies are key mediators of allergic reactions. Due to their potentially harmful anaphylactic properties their production is tightly regulated. The membrane-bound isoform of IgE (mIgE), which is an integral component of the B cell antigen receptor, has been shown to be critical for the regulation of IgE responses in mice. In primate species including humans, mIgE can be expressed in two isoforms that are produced by alternative splicing of the primary ϵ Ig heavy chain transcript, and differ in the absence or presence of an extracellular membrane-proximal domain (EMPD) consisting of 52 amino acids. However, the function of the EMPD remains unclear. Here, we demonstrate that the EMPD restricts surface expression of mIgE-containing BCRs in human and murine B cells. The EMPD does not interfere with BCR assembly but acts as an autonomous endoplasmic reticulum retention domain. Limited surface expression of EMPD-containing mIgE-BCRs caused impaired activation of intracellular signaling cascades and hence

represents a regulatory mechanism that may control the production of potentially anaphylactic IgE antibodies in primate species.



Owing to alternative splicing of primary transcripts, B cell antigen receptors of the mIgE isotype can be expressed as short or long isoform in human and primate B cells. We demonstrate that the long mIgE isoform contains an ER retention domain, which limits its capacity to initiate signal transduction.

Introduction

The production of antibodies by B cells is initiated following recognition of antigen by the B cell antigen receptor (BCR) complex. Stimulation of the BCR activates a complex network of intracellular signaling pathways that along with costimulatory signals drives cellular activation and differentiation towards antibody-producing plasma cells [1, 2]. In the course of an antibody response, some B cells switch their immunoglobulin (Ig) isotype from IgM to other Ig classes in a T helper (Th) cell-dependent manner primarily during germinal center reactions. Ig class-switch recombination to IgE occurs in an interleukin-4-driven manner during Th2 cell-dominated immune responses [3]. Whereas soluble IgE antibodies have gained much attention due to their key role in allergic reactions such as asthma or atopic dermatitis [4, 5], the membrane-bound form of IgE (mIgE) is less well studied. Like other mIg isotypes, mIgE is expressed as part of the BCR complex together with the signaling subunits Ig α (CD79a) and Ig β (CD79b) on the B cell surface [6, 7]. The Ig α / β heterodimer initiates BCR signaling by recruiting and activating cytoplasmic tyrosine kinases to immunoreceptor tyrosine-based activation motifs (ITAMs) [8, 9] and by recruitment of the central B cell adapter protein SLP65 (BLNK) to a non-ITAM tyrosine in Ig α [10-12]. Phosphorylation of SLP65 by the BCR-associated protein tyrosine kinase Syk creates a signaling hub termed 'Ca²⁺ initiation complex', from which the production of second messengers by phospholipase C- γ 2 (PLC- γ 2) and the subsequent activation of ubiquitous signaling pathways such as the Erk MAP kinase cascade or the PI3' kinase/Akt pathway are initiated. Furthermore, signaling of mIgE- (and mIgG-) containing BCRs is enhanced by tyrosine-based signaling motifs in the cytoplasmic domains of mIgE (and mIgG), termed immunoglobulin tail

tyrosine (ITT), that recruit the ubiquitous adapter protein Grb2 into the BCR signalosome [13, 14].

Our knowledge on the biology of mIgE-expressing B cells has been significantly expanded in recent years due to the development of genetic mouse model systems that allow tracking and functional analysis of this scarce cell population [15-18]. In particular, the genetic analysis of the mIgE-BCR in mouse B cells revealed that expression of mIgE at a certain time point in the life of an IgE-switched cell is critical for the subsequent production of soluble IgE antibodies, since selective genetic deletion of the membrane-bound form of IgE causes an almost complete lack of serum IgE *in vivo* but not *in vitro* [19]. Furthermore, two recent studies described unique intrinsic properties of murine mIgE that seem to regulate the fate of IgE-switched cells, in that mere expression of mIgE drives their rapid differentiation into IgE-secreting plasma cells and/or induces apoptosis of the cells in an antigen-independent manner [20, 21]. Collectively, these studies showed that the mIgE-containing BCR is a critical regulator of IgE responses *in vivo*.

In contrast to mouse mIgE, human and primate mIgE can be expressed in two isoforms that are generated by alternative splicing of primary ϵ Ig heavy chain transcripts [22-24]. The two isoforms are distinguished by the presence or absence of 52 amino acids between the most C-terminal constant Ig domain (CH4) and the transmembrane domain (Fig. 1A). This region is referred to as extracellular membrane-proximal domain (EMPD, alternatively called C ϵ mX), and is encoded by the so-called M1' exon (alternatively called *me.p*). The different splice acceptor sites that allow for expression of the two isoforms of human mIgE are conserved in several primate species but not in other mammals tested [25]. The resulting mIgE isoforms are referred herein as long (EMPD-containing) and short (EMPD-deficient) mIgE,

respectively. The usage of the two isoforms by human IgE-switched B cells is poorly characterized. Whereas some reports showed that the long isoform is far more abundant than the short isoform in mIgE-expressing B cell lines [22, 23], others found transcripts for the short isoform in peripheral blood B cells as well [24]. Functional comparisons of the two mIgE isoforms suggested that the EMPD might influence BCR signaling and thereby regulate activation induced cell death *in vitro* [7]. However, the molecular mechanisms that underlie these observations as well as the signaling pathways that are affected by the EMPD remained unclear.

In this study we compared the subcellular localizations as well as the signaling capabilities of the long and short mIgE isoform in human and murine B cells. We show that the EMPD efficiently suppresses surface expression of the long mIgE isoform by retaining it in the endoplasmic reticulum, a function that is conserved among primate species. The inefficient surface expression of long mIgE-BCRs drastically restricted their signaling capacity. Hence the EMPD acts as a BCR-intrinsic inhibitory domain of intracellular signal initiation. These findings suggest that the different mIgE isoforms of primate species regulate the biology of IgE-switched B cells and thus may control the production of IgE antibodies.

Results

The EMPD restricts cell surface expression of mIgE-BCRs

To investigate the different isoforms of human mIgE-BCRs, we generated retroviral expression vectors for the long (EMPD-containing) and short isoform of the human membrane-bound ϵ (ϵ m) Ig heavy chain and expressed both variants in the human B cell line

DG75. Flow cytometric analysis revealed a striking difference in the amount of mIgE on the cell surface in that the short isoform was approximately four to five times more abundant in the plasma membrane than the long isoform (Fig. 1B, left histograms & Fig. 1C). Staining of mIgE in permeabilized cells revealed that the total amounts of long and short mIgE in the cells were comparable (Fig. 1B, right histograms & Fig. 1C). We consistently observed mIgE-negative populations among the transfected DG75 cells, the proportions of which were similar for all constructs and which were excluded from the analyses by gating. We also tested the expression of the ϵ m Ig heavy chain isoforms by western blot analysis of cellular lysates, which confirmed the flow cytometry data (Fig. 1D). Note that the short but not the long ϵ m Ig heavy chain variant runs as a double band. The higher molecular weight form (indicated by an arrowhead) represents the pool of fully glycosylated cell surface-bound ϵ m Ig heavy chain (Fig. 3 and [7]).

Previous studies of human mIgE isoforms were carried out in different mouse B cell lines [7, 26]. In addition the human EMPD coding sequence has been integrated into the mouse genome to create EMPD-containing mouse/human chimeric mIgE-BCRs [16, 27]. To test if our observations made in the human B cell line DG75 also apply to murine cells, we retrovirally transfected primary splenic mouse B cells with constructs encoding short or long human ϵ m Ig heavy chains along with an IRES-EGFP cassette, and tested the expression of human mIgE isoforms in EGFP-positive cells as before (Fig. 1E). We observed the same striking difference in cell surface deposition of the two mIgE isoforms, despite comparable expression of both proteins within the cells. In conclusion these results indicated that the EMPD in the long isoform of human mIgE restricts surface expression of human mIgE-BCRs in human as well as murine B cells.

An EMPD of 52 amino acids is not specific for humans but is found in several primate species yet not in other mammals tested including the mouse [25]. To test whether the function of the EMPD is conserved during primate evolution, we replaced the human EMPD with that of two distantly related primate species, the ape *Hylobates lar* (Lar gibbon) as well as the New World monkey *Ateles geoffroyi* (Geoffroy's spider monkey) (Fig. 1F). We chose the EMPDs of these species since their amino acid sequences differ significantly from the human EMPD sequence with ~81% sequence identity for *Hylobates lar* and ~85% for *Ateles geoffroyi* and furthermore because they differ substantially among each other (~71% sequence identity). Interestingly, New World monkeys exclusively express the long isoform of mIgE since they lack a splice acceptor site that is required to allow for alternative splicing to make the short mIgE isoform [25]. Expression of the chimeric long mIgE variants containing the EMPDs of the two primate species in DG75 B cells showed that both EMPDs restrict cell surface expression of mIgE-BCRs to a similar extent as the human EMPD, despite being expressed within the cells in similar amounts (Figs. 1G & H). Hence, restriction of cell surface expression of mIgE-BCRs is an evolutionary conserved function of the mIgE-EMPD.

The long mIgE-BCR is predominantly localized in the endoplasmic reticulum

To investigate the subcellular localization of mIgE in more detail, we stained the two human mIgE isoforms in permeabilized cells and analyzed their distribution in the cell by confocal microscopy. Figure 2A shows representative series of z-stack frames of DG75 cells expressing either the short (S) or the long (L) isoform of human mIgE stained with an anti-IgE antibody. The mIgE fluorescence signal intensities in the equator planes of the cells indicated that the short isoform of mIgE is localized in 2 concentric structures, most likely

representing the cell surface and the endoplasmic reticulum (ER) and probably some trafficking organelles. However, the long mIgE isoform was predominantly found in one perinuclear structure, suggesting it to be localized mainly in the ER (Fig. 2B). To further characterize the subcellular distribution of the two mIgE isoforms, we co-stained them with an antibody against Calnexin to reveal the ER, wheat germ agglutinin to mark membranes and DAPI to stain nuclei (Fig. 2C). Whereas the short isoform of mIgE was localized at both the ER and the plasma membrane, long mIgE mainly co-localized with the Calnexin signal in a perinuclear pattern and only a minor fraction was found on the cell surface (Fig. 2C). Consistent with the flow cytometry data, the analysis of >150 cells by confocal microscopy revealed a four-fold lower plasma membrane localization of the long mIgE isoform compared with the short isoform (Fig. 2D), indicating that the EMPD might interfere with the transport of long mIgE to the cell surface.

The EMPD does not interfere with BCR assembly

It is well established that membrane-bound immunoglobulins need to associate with their signaling subunits Ig α and Ig β in order to get efficiently transported to the cell surface [6, 28, 29]. Hence, impaired assembly of long mIgE with Ig α / β to form a functional BCR represented a possible explanation for its ER localization. To test this scenario, we affinity-purified the two mIgE isoforms from different cellular compartments of DG75 B cells and detected the amount of co-purified Ig α / β by Western blot analysis (Fig. 3A). Specifically, we affinity-purified mIgE from the cell surface (S) and used the remaining lysates for affinity purification of the intracellular pool (IC) of mIgE. In addition, we used cellular lysates to purify the total pool (T) of mIgE from the cells. Clearly, co-purification of Ig α / β with the long

mIgE isoform was not impaired compared with the short isoform (Fig. 3A). As a matter of fact, Ig α / β was co-purified much more efficiently with the long mIgE isoform than with the short variant, especially from intracellular compartments. Hence, we conclude that impaired assembly of BCR complexes in the ER is not responsible for the inefficient surface expression of the long mIgE isoform. Figure 3A furthermore showed that the surface pool of short ϵ m ran at a higher molecular weight than the intracellular pool (see also Fig. 1D), which was most likely due to different and more complex glycosylation of the mature surface expressed pool of short mIgE. The cell surface pool of immunoglobulins (and other transmembrane proteins) is generally resistant to de-glycosylation with EndoH but not PNGaseF, whereas ER-resident pools of immunoglobulins can be de-glycosylated with both enzymes. Indeed the cell surface-bound pool of short mIgE was resistant to de-glycosylation with EndoH (Fig. 3B, lane 5, black arrowhead). Furthermore there was a large pool of short mIgE purified from total cells that was resistant to treatment with this enzyme (lane 6, black arrowhead). In contrast the long isoform was (almost) completely de-glycosylated by EndoH (lane 8, grey arrowhead), leaving only a minute pool of EndoH-resistant long ϵ m (lane 8, open arrowhead). Even the surface-bound pool of mIgE was de-glycosylated by EndoH and ran as a crisp band (lane 7, grey arrowhead), indicating that the small pool of long mIgE that is found in the plasma membrane has escaped full glycosylation in the Golgi apparatus. Please note that the signal of glycosylated cell surface-expressed long ϵ m is very faint and fuzzy and hence cannot easily be visualized by Western blotting. As control we treated the samples with PNGaseF, which caused full de-glycosylation of both ϵ m isoforms. These biochemical data corroborate the notion that the long mIgE isoform is efficiently retained in the endoplasmic reticulum and usually does not reach distal Golgi compartments.

The EMPD is an autonomous ER retention domain

Since the EMPD did not prevent surface expression of mIgE by interfering with BCR assembly, we reasoned that it might serve as a domain that mediates retention of the long mIgE isoform within the ER. If the EMPD represents an autonomous ER-retention domain, it should be able to exert its function outside of the BCR complex, i.e. within a different polypeptide backbone. To test this notion, we introduced the EMPD into a chimeric receptor, consisting of the extracellular and transmembrane domains of human CD8 α fused to the cytoplasmic tail of Ig α that in addition contained a C-terminal HA-tag (CD8-EMPD/Ig α -HA) (see Fig. 4A). Whereas the EMPD-deficient variant of this chimera was readily expressed on the cell surface of human B cells, the EMPD-containing counterpart was undetectable by surface staining (Fig. 4B, left histograms & Fig. 4C). Staining of permeabilized cells (Fig. 4B, right histograms & Fig. 4C) as well as Western blot analysis of cellular lysates (Fig. 4D) proved that both chimeras were expressed in similar amounts in the cells. In summary, the results show that the EMPD of human and other primates' mIgE is an ER-retention domain that functions autonomously in different polypeptide backbones.

The EMPD restricts the signaling capacity of mIgE-BCRs

Having established that the EMPDs of primate species inhibit surface expression of mIgE-BCRs, we asked whether this effect was associated with altered mIgE-BCR signaling. Activation of the Ca²⁺ initiation complex was tested by affinity purification of PLC- γ 2 from lysates of anti-IgE stimulated cells (Fig. 5A). Both, phosphorylation of PLC- γ 2 (Fig. 5A, upper panel) as well as its complex formation with SLP65 (middle panel) were reduced and very transient in cells expressing the long isoform of human mIgE compared with cells expressing

the short mIgE variant. In accordance with these findings, analysis of BCR-induced mobilization of the second messenger Ca^{2+} showed that the long mIgE-containing BCR initiated only a transient Ca^{2+} flux (Fig. 5B, left graph, red curve), whereas the Ca^{2+} response of short mIgE-expressing cells (Fig. 5B, left graph, blue curve) was much stronger and longer lasting. To test the general signaling capacities of the transfected cells, we analyzed Ca^{2+} mobilization upon stimulation of the endogenous DG75 mIgM-BCRs, which was identical for both transfectants (Fig. 5B, right graph). In the same way, we tested the Ca^{2+} responses of cells expressing our chimeric mIgE-variants containing the EMPDs from *Hylobates lar* (HL-EMPD) or *Ateles geoffroyi* (AG-EMPD). Again, the EMPD-containing long isoforms of mIgE showed a much weaker capacity to activate PLC- γ 2-controlled Ca^{2+} mobilization (Fig. 5C).

The inefficient activation of BCR-proximal signaling events by the long mIgE-BCR isoform also translated into a strongly reduced activation of downstream pathways such as the Erk MAP kinase cascade (Fig. 6A). Likewise, activation of the PI3' kinase pathway was reduced in cells stimulated via the long mIgE isoform, as assessed by inefficient phosphorylation of the B cell co-receptor CD19 (Fig. 6B) and the PI3' kinase-regulated serine/threonine kinase Akt (Fig. 6C). In conclusion the EMPD strongly restricts the capacity of mIgE-BCRs to activate proximal and distal signaling pathways that are critical for B cell activation.

Both mIgE isoforms stimulate activation-induced cell death

Previous studies reported that human mIgE-containing BCRs have the capacity to induce apoptosis (or activation-induced cell death) in different mouse B cell lines [7, 26]. In these experiments EMPD-containing receptor variants were less capable of inducing apoptosis than EMPD-deficient constructs. We tested the capacity of both human mIgE isoforms to

induce apoptosis in DG75 B cells by stimulating the receptors with anti-IgE antibodies for 24 and 48 hours. As control we stimulated the endogenous mIgM-BCR of DG75 cells. The results show that both mIgE-BCR isoforms have the capacity to induce apoptosis with the short isoform being somewhat more efficient when analyzed after 24 hours, which might reflect the enhanced signaling capacity of the short mIgE-BCR (Fig. 7A & B). However, when analyzed after 48 hours of stimulation there was no detectable difference in the capacity to induce apoptosis between the two mIgE-BCR isoforms (Fig. 7B). Also there was no obvious difference between mIgE-BCRs and endogenous mIgM-BCRs. In conclusion, both isoforms of human mIgE have the capacity to stimulate activation-induced cell death in DG75 B cells. However, whether this also occurs *in vivo* in primary human B cells that have undergone Ig class-switching to IgE in a germinal center where there are co-stimuli provided to the B cells remains to be investigated.

Discussion

The production of soluble IgE antibodies *in vivo* critically depends on expression of mIgE-containing BCRs on the surface of B cells that have undergone Ig class-switching to the ϵ locus [19]. Furthermore, recent findings using *in vitro* cultures of primary mouse B cells suggest that signals emanating from the mIgE-BCR regulate the terminal differentiation of mIgE-expressing cells into plasma cells in an antigen-independent manner [20, 21]. In line with this argumentation, expression of mIgE-BCRs on murine post germinal center (GC) B cells seems to predispose the cells to differentiate towards the plasma blast stage *in vivo* [15]. Together, these findings demonstrate that the expression of mIgE-BCRs on the B cell surface controls the differentiation fate of IgE-switched cells and the subsequent production

of serum IgE in the mouse. Even though there are no genetic data available that directly demonstrate a similar role for mIgE in humans, mutations that prevent surface expression of mIgM or mIgG2 cause either total or isotype-specific antibody deficiencies in human patients [30, 31]. Thus, it appears likely that surface expression of mIgE is a critical determinant for the production of IgE antibodies also in humans, e.g. in allergic reactions. Unlike murine B cells, human (and Old World monkey) B cells can regulate the amount of cell surface-expressed mIgE by choosing between two splice variants. Whether there is a bias for a certain isoform in primary human B cells is not known, however, in *in vitro* cultures of primary human B cells, expression of the short isoform was associated with efficient plasma cell differentiation [32], which once more indicates that differential usage of the two mIgE isoforms may regulate the production of IgE antibodies. Interestingly, New World monkeys have lost the ability to express the short mIgE isoform altogether. Whether this goes along with altered production of soluble IgE antibodies in these animals is not known. However, when the human M1' exon was introduced into the murine ϵ Ig locus to generate a mouse/human chimeric ϵ m Ig heavy chain containing the human EMPD, this chimeric mIgE molecule was poorly expressed on the cell surface [16], which can be explained by the results presented herein. Accordingly, these mice whose B cells exclusively expressed the long isoform (like B cells of New World monkeys) showed approximately six-fold reduced serum titers of IgE antibodies when compared with wild type mice [16]. Very recently another knock-in mouse expressing a similar EMPD-containing mouse/human ϵ m heavy chain was described, which showed a seven-fold reduction of serum IgE titers [27]. Hence, we speculate that in primates (including humans) the long isoform of mIgE has evolved as a negative regulatory element of IgE antibody production.

Thus far, little was known about functional differences between the two isoforms of human mIgE. Initial experiments showed that both isoforms associate with the Ig α / β signaling device, yet seemed to deliver qualitatively different signals into the cell [7]. However, both isoforms were expressed on the surface of transfected mouse B cells in similar amounts [7]. Although we cannot resolve this discrepancy to our findings, a possible explanation might be that selection of clones with similar surface expression was done in these early experiments whereas we used polyclonal populations of retrovirally transduced cells. Be that as it may, by analyzing the EMPD from different primate species and by transplanting the human EMPD into a different polypeptide backbone we showed that it fulfills an evolutionary conserved function, which is to act as an autonomous ER-retention domain that restricts surface expression and thus signaling of mIgE-BCRs. Even though we cannot formally exclude that ER-resident (long) mIgE exerts some kind of signaling activity, such a scenario appears very unlikely since previous studies have shown that ER-located fully assembled pre-BCR complexes are not associated with BCR-proximal tyrosine kinases and hence do not initiate signaling [33, 34].

The mechanisms that govern localization of transmembrane proteins in the ER or their shuttling between ER and Golgi apparatus, respectively, are only partially understood [35]. Probably the best-studied mechanism involves a carboxyl-terminal, i.e. cytosolic, -KKXX or -KXXXX (where X is any amino acid) di-lysine motif found in many ER-resident transmembrane proteins [36-38]. Alternatively, hydrophilic amino acids within otherwise hydrophobic transmembrane regions have the potential to confer ER retention through an ill-defined mechanism [39]. In addition, monomeric soluble IgM molecules can be retained in the ER via a C-terminal cysteine residue [40]. In case of the mIgE-EMPD the retention motif is located within the extracellular/ER-luminal part of the molecule. As there are only

few examples in the literature describing extracellular ER retention motifs in transmembrane proteins, we can only speculate that the mIgE-EMPD mediates a stable interaction with some ER-resident component, probably a chaperone that might recognize the EMPD as an intrinsically unfolded domain and thus prevent ER exit of the long mIgE variant [35]. This notion is supported by a recent report, which indicates that the EMPD is an intrinsically disordered domain that does not adopt ordered secondary structures [41]. Since the folding process of immunoglobulins is accompanied and tightly controlled by the action of ER-resident chaperones such as heavy chain binding protein (BiP, HSPA5) [42], the presence of an unfolded domain in the long ϵ m Ig heavy chain might not be tolerated by the ER quality control system. Nevertheless, further research is needed to clarify the molecular details by which the EMPD is trapped within the ER. We conclude that the EMPD acts as receptor-intrinsic domain to restrict signaling of the mIgE-BCR. This regulatory mechanism may have evolved in primates (including humans) to curb the production of IgE antibodies, probably because of the powerful and potentially life-threatening anaphylactic properties of this antibody isotype.

Materials and methods

Cells and Expression Vectors

The human Burkitt lymphoma line DG75 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and was maintained in RPMI 1640 + 10% fetal bovine serum and antibiotics. The cells were transfected to express the murine cationic amino acid transporter 1 (SLC7A1) to make them susceptible for infection with MMLV-based retrovirus particles and were selected with blasticidin S (10

µg/ml). The resultant DG75EB cells were used in all experiments. The cDNA encoding the long isoform of the membrane-bound human ϵ immunoglobulin heavy chain, amplified from the human IgE-secreting myeloma line U266, was a gift of Dr. Jürgen Frey, University of Bielefeld, Germany. The V region of that cDNA was replaced by the NP-specific B1-8 sequence containing an intron via an endogenous PshAI restriction site. To generate the short ϵ m isoform the 52 codons of the EMPD were deleted by mutagenesis PCR. The chimeric CD8-(EMPD)/Ig α -HA constructs were synthesized by Eurofins Genomics (Ebersberg, Germany). The integrity of all constructs was confirmed by DNA sequencing. All cDNAs were ligated into pMSCVpuro (Clontech) and retroviral infections were done using the Plat-E packaging cell line. Infected DG75EB cells were selected in the presence of 2 µg/ml puromycin. Primary mouse B cells were isolated from spleens of 9-11 weeks old male BALB/c mice using magnetic cell sorting (mouse B cell isolation kit, Myltenyi Biotec, Bergisch Gladbach, Germany), which gave rise to >95% CD19-positive cells. Retroviral gene transfer of human ϵ m immunoglobulin heavy chain isoforms into LPS-activated primary splenic mouse B cells using the MIGR11 plasmid, which contains an IRES-EGFP cassette was done as described before [13].

Biochemical and cellular assays

For BCR stimulation, cells were incubated with 10 µg/ml goat anti-human IgE antibodies (Abcam) or goat F(ab')₂ fragments against human IgM (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK) at 37°C in RPMI for the indicated times. For induction of apoptosis cells were stimulated with the indicated antibodies for 24 and 48 hours in RPMI + 5% FCS at 37°C. Preparation of cellular lysates in 1% NP40-containing lysis buffer for affinity

purifications and western blot analyses was done as described [10]. Biotinylated anti-human IgE (Thermo Fisher Scientific) and streptavidin sepharose (GE Healthcare) were used for purification of mIgE-BCRs. Biotinylated anti-CD19 (clone HIB19, BioLegend) was used to affinity purify CD19. Anti-phospho-Erk (Thr 202/Tyr 204, clone E10), anti-phospho-Akt (Ser 473, clone D9E), anti-Akt (rabbit polyclonal), anti- β -Actin (clone 13E5) and anti-CD19 (rabbit polyclonal) for western blotting were from Cell Signaling Technology. Anti-phospho-tyrosine antibodies were from Upstate Biotechnology (clone 4G10) and Cell Signaling Technology (clone 100), respectively. Antibodies against Ig α (clone EP3618) and Ig β (clone EPR6861) were from Abcam. Anti-PLC- γ 2 antibody (Q20) was from Santa Cruz, anti-SLP65 (clone 2C9) from BAbCo, anti-pan Erk (clone 16/ERK) from BD Biosciences and anti-HA (clone 3F10) from Roche. Quantification of western blot band intensities was done using Image J software and Microsoft Excel.

Flow Cytometry

Goat anti-human IgE was used in combination with Alexa-Fluor 647-conjugated donkey-anti-goat IgG (both from Abcam). Biotin-conjugated anti-human CD8 (clone MEM31 from Exbio, Czech Republic) was used in combination with Alexa Fluor 647-conjugated streptavidin (BioLegend). For staining of intracellularly localized molecules, cells were fixed in Cytofix buffer (BD Biosciences), followed by permeabilization and staining in Phos-flow Perm/Wash buffer I (BD Biosciences). Untransfected DG75EB cells were used as negative control. Staining of early and late apoptotic cells was done with AnnexinV-BV421 (BioLegend) and 7-AAD (BD Biosciences).

Ca²⁺ measurements

Analysis of BCR-induced intracellular Ca²⁺ flux with Indo1-AM was done as described [13]. To avoid a falsifying influence of the mIgE-negative cells in our transfectants on the Ca²⁺ flux results, non-responding cells were excluded from the analysis by applying a minimum Indo-1 fluorescence ratio threshold.

Microscopic analyses

Cells were seeded on a poly-L-lysine-coated glass coverslip for 60 minutes at 37°C before they were fixed with 4 % paraformaldehyde (PFA) for 30 minutes at room temperature. Subsequently, residual PFA was quenched with 100 mM Glycine in PBS and washed away with PBS. Membrane staining was performed with wheat germ agglutinin (WGA) coupled to Alexa Fluor 594 (Thermo Fisher Scientific) diluted 1:200 in PBS for 10 minutes at room temperature. Cells were then permeabilized with PBS supplemented with 0.05% Triton X-100 for 1 minute and followed by a blocking step using PBS supplemented with 1.5 % bovine serum albumin for 30 minutes at room temperature. Labeling of mIgE, Calnexin and nuclei was done using the following reagents: 1:100 dilution of biotin-labeled anti-human IgE (Thermo Fisher Scientific), 1:100 dilution of rabbit anti-Calnexin (clone C5C9, Cell Signaling Technology), 1:1,000 dilution of Alexa Fluor 488-coupled streptavidin, 1:1,000 dilution of Alexa Fluor 647-coupled donkey-anti-rabbit IgG and DAPI (all from Molecular Probes). Laser Scanning Confocal microscopy was performed using a True Confocal System SP5 fluorescence microscope (Leica Microsystems, Mannheim, Germany) equipped with a 1.4 NA/100x objective. Pixel size was set to 60.5 nm and the scanning speed to 1 kHz. Signals were detected with independent photomultipliers (PMTs) for each color line. Excitations were

performed with a 488 nm argon, a 543 nm helium and a 633 nm helium/neon laser. DAPI nuclei were excited using a MaiTai pulsed tunable laser at 800 nm (Mai Tai Broadband, Spectra-Physics, Santa Clara, CA, USA). Analysis of fluorescence signal distribution was performed manually using ImageJ software. Freehand lines were manually drawn as region of interest (ROI) with a thickness of 3 pixels following the Calnexin (ER) signals to calculate the average fluorescence intensities from the mIgE signals. Similarly, ROIs were drawn following the WGA (plasma membrane) signals and used as a mask to calculate the average intensities of the cell surface mIgE signals. An unpaired two-tailed student's t-test with 95% confidence interval was performed with Prism Inc. software.

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Conflict of interest:

The authors declare no financial or commercial conflict of interest.

Figure legends

Figure 1. (A) Schematic representation of the short (S) and long (L) isoforms of human mIgE. The EMPD in long mIgE is colored in red. (B) DG75 cells expressing either the short (S, blue curves) or long (L, red curves) variant of human mIgE were analyzed for expression of surface-bound mIgE (left panel) or total mIgE (right panel) by flow cytometry. Untransfected cells were used as controls (filled grey curves). Gating on positive populations is indicated by dashed vertical lines. (C) Normalized mean fluorescence intensities (MFI) pooled from three independent experiments shown in (B). The values of the short mIgE isoform were set to 1.0. All other values were normalized accordingly. Error bars represent standard deviation. (D) Cell lysates were analyzed for expression of the respective ϵ m Ig heavy chain (HC) by immunoblot analysis with anti- ϵ antibodies (upper panel) or anti- β -Actin as loading control (lower panel). S: short, L: long. The arrowhead indicates the fully glycosylated surface-bound form of the short human ϵ m HC. Data are representative of three independent experiments. (E) LPS-activated primary splenic mouse B cells were retrovirally transduced to express human ϵ m isoforms along with EGFP. EGFP-positive cells were analyzed for expression of mIgE as in (B) and (C). Data are pooled from three independent experiments. (F) EMPD amino acid sequence alignment of the indicated species was done using the ClustalW program. Amino acids in red indicate residues that differ from the human sequence. (G & H) Expression of the short (S) isoform of human mIgE and the indicated chimeras was analyzed as in (B) and (C).

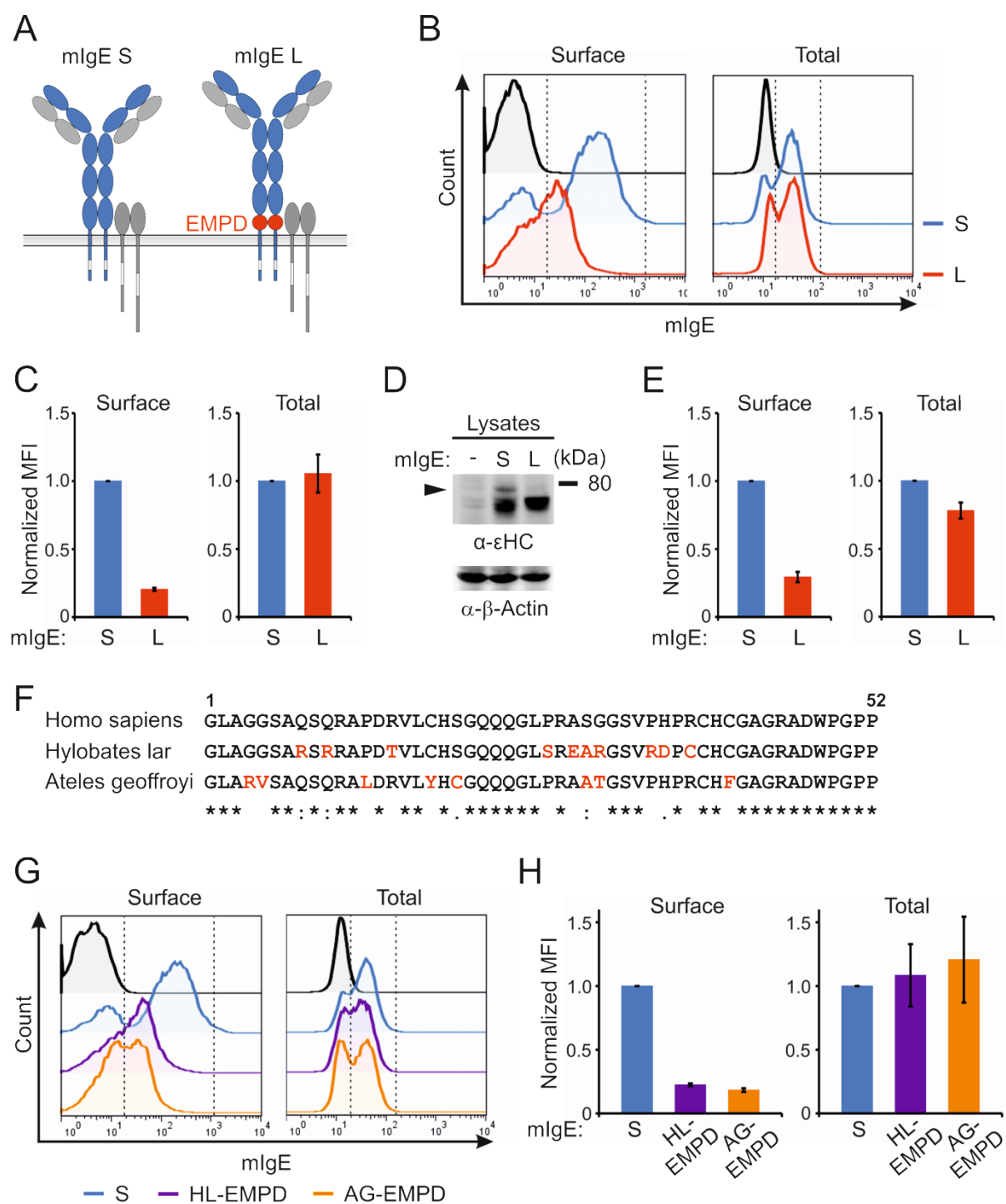


Figure 1

Figure 2. (A) Fixed and permeabilized DG75 cells expressing either the short (S) or long (L) isoform of human mlgE were stained with anti-IgE and analyzed by confocal microscopy. Confocal Z-stack images (of ~0.9 μm steps) were obtained to collect the mlgE signal distribution throughout the cells. The mlgE intensities at the cellular equator (indicated by white asterisks) are represented as a 3D surface plot in (B) Scale bar: 5 μm . (C) Confocal images of cells co-stained with anti-IgE (green), anti-Calnexin (ER, red), wheat germ agglutinin (magenta) and DAPI (blue; only in Merge). A line profile of the relative fluorescence intensities through the different stained structures was obtained along the white arrows. Scale bar: 2 μm . (D) Statistical analysis of the fluorescence intensities for short (S) and long (L) mlgE in the plasma membrane (surface) relative to those in the ER was calculated from a total of 76 (S) and 84 (L) cells. All data are representative of three independent experiments, each including two technical replicates. 25-30 cells were analyzed per experiment. Statistical significance was calculated using unpaired t-Test, **** $p < 0.0001$.

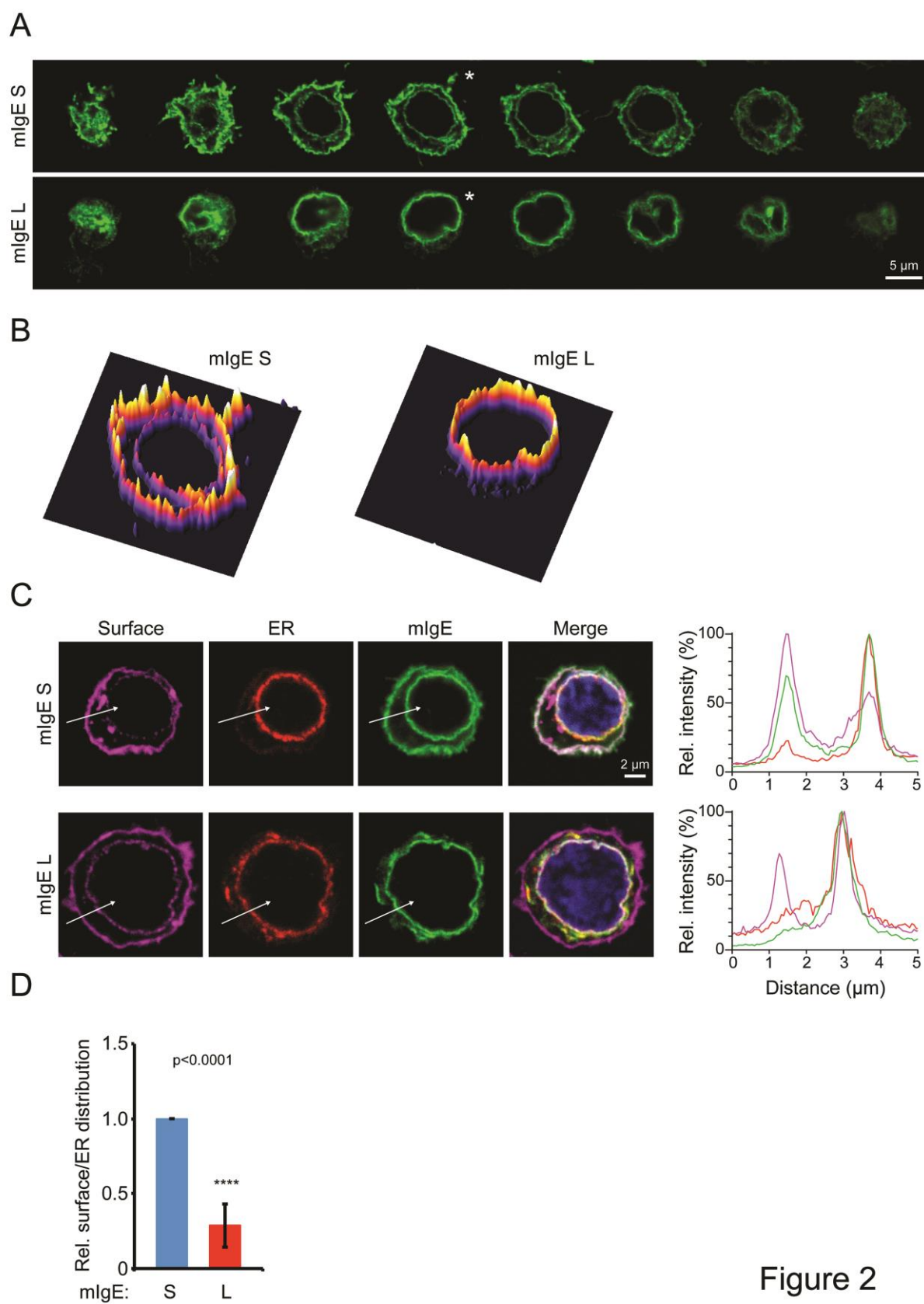


Figure 2

Figure 3. (A) Affinity purification (AP) of mIgE-containing BCRs was done using biotinylated anti-human IgE antibodies and streptavidin sepharose from cells that had been labeled with the antibodies before lysis to purify the cell surface pool (S) of mIgE. Following the AP, the remaining lysates were used to purify the intracellular pool (IC) of mIgE molecules. Cleared cellular lysates were used to purify the total pool (T) of mIgE molecules. All purified proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and consecutively analyzed by immunoblotting with antibodies to the ϵ Ig heavy chain (ϵ HC, upper panel), Ig α (middle panel) and Ig β (lower panel). Parental DG75 cells that did not express any mIgE served as negative control. Data are representative of three independent experiments. (B) Affinity-purified mIgE isoforms from the cell surface (S) or total cell lysates (T) were analyzed as in (A) (lanes 1-4) or were treated with EndoH (lanes 5-8) or with PNGaseF (lanes 9-12) before they were subjected to SDS-PAGE and Western Blot analysis. Arrowheads indicate the positions of glycosylated or de-glycosylated ϵ m immunoglobulin heavy chains (see text for details). Data are representative of three independent experiments.

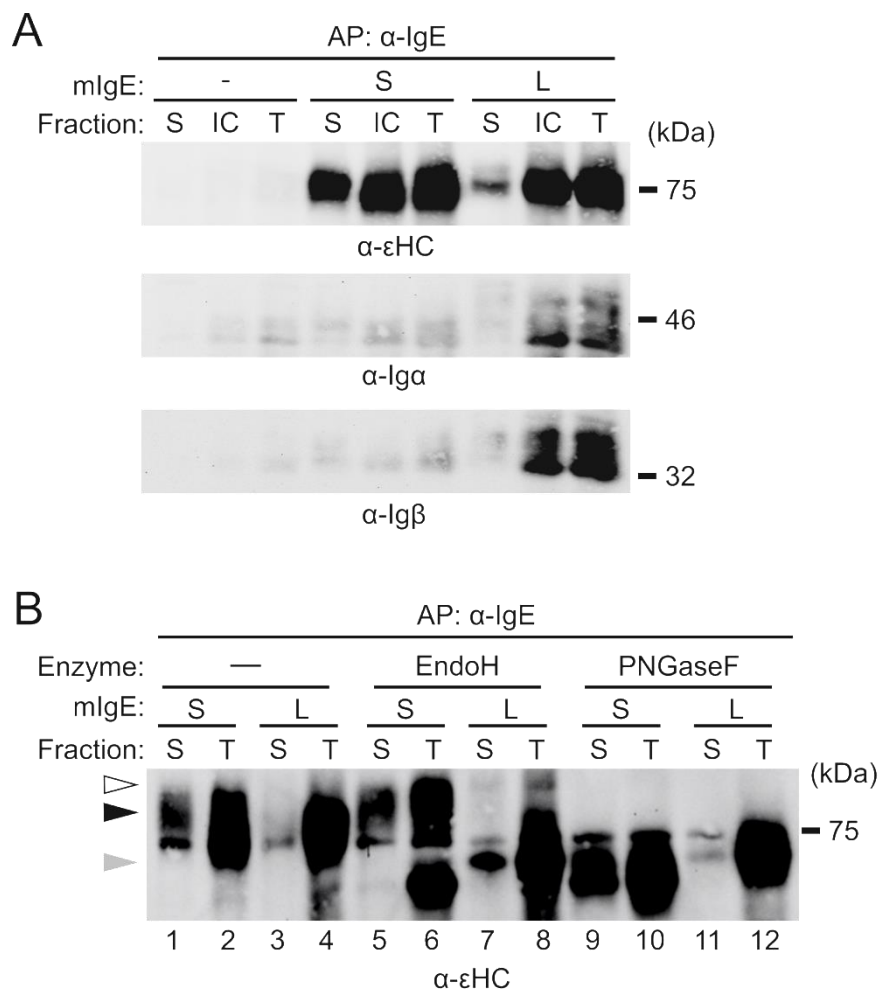


Figure 3

Figure 4. (A) Amino acid sequence alignment of the short human ϵ m Ig HC and human CD8 α . Shown is the region between the most C-terminal constant immunoglobulin domain (CH) of ϵ m and the transmembrane domains (TM). The sequence and position of the EMPD in human ϵ m and in the chimeric CD8/Ig α -EMPD-HA molecule is indicated by the purple line. Alignment was done using the ClustalW program followed by manual correction. (B) DG75 cells expressing either CD8/Ig α -HA (-EMPD, blue curves) or CD8-EMPD-Ig α -HA (+EMPD, red curves) were analyzed for cell surface expression of the respective molecules (left histograms) or total expression in permeabilized cells (right histograms). Untransfected cells were used as controls (filled grey curves). Gating on positive populations is indicated by dashed vertical lines. (C) Relative expression of the cell surface pool and total pool of the different CD8 chimeras was analyzed as in Fig. 1. Data are pooled from three independent experiments as in Fig. 1. Error bars represent standard deviation. (D) Cell lysates were analyzed for expression of the respective CD8 chimeras with by immunoblot analysis with anti-HA antibodies (upper panel) or anti- β -Actin antibodies as loading control (lower panel). The arrowhead indicates the fully glycosylated surface-bound form of CD8/Ig α -HA. Data are representative of two independent experiments.

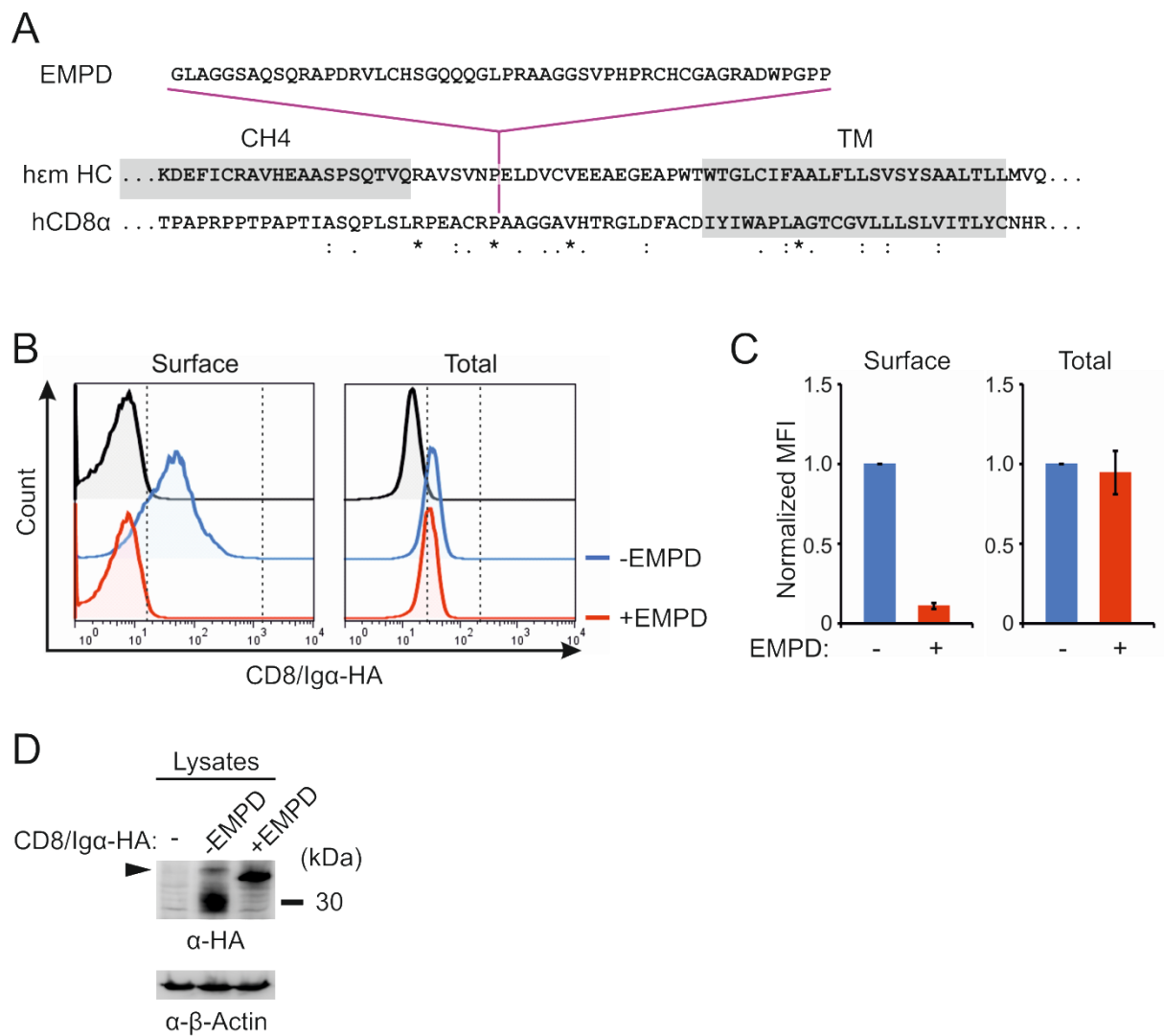


Figure 4

Figure 5. (A) Analysis of the Ca^{2+} initiation complex in cells expressing short or long mIgE-BCRs. Phospholipase C- γ 2 (PLC- γ 2) was affinity purified (AP) from lysates of anti-IgE stimulated cells and subjected to immunoblot analysis with antibodies to phospho-tyrosine (upper panel), SLP65 (middle panel) and PLC- γ 2 as loading control (lower panel). (B) Ca^{2+} mobilization in the same cells was analyzed using Indo-1-AM. Baseline Ca^{2+} levels were monitored for 30 seconds, following addition of stimulating anti-IgE or anti-IgM antibodies (indicated by arrows). Data are representative of three independent experiments (C) Analysis of BCR-induced Ca^{2+} mobilization in cells expressing the short isoform of human mIgE or the indicated chimeras containing the EMPDs from either *Hylobates lar* (HL-EMPD) or *Ateles geoffroyi* (AG-EMPD). Data are representative of three independent experiments.

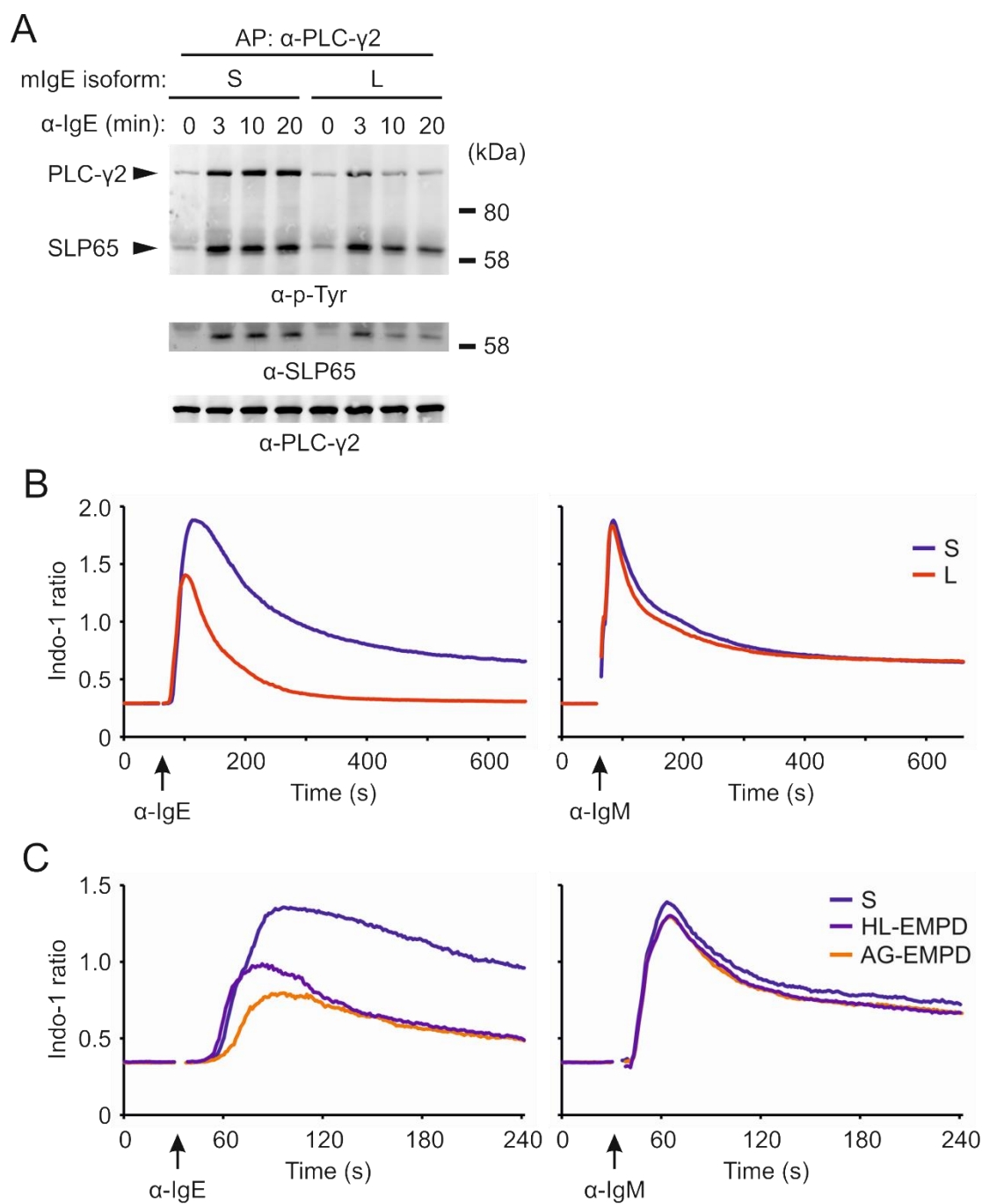


Figure 5

Figure 6. (A) Activation of the Erk MAP kinase in anti-IgE-stimulated cells was analyzed by immunoblot analysis of cellular lysates with anti-phospho-Erk (p-Erk) antibodies (upper panel). The blot was subsequently developed with anti-Erk antibodies (lower panel) as loading control. The ratio of band intensities for phospho-Erk relative to the total Erk signals is given below each lane. The graph shows quantification of normalized band intensities summarized from three independent experiments. Blue curve: data points for cells expressing short mIgE (S), red curve: data points for long mIgE (L). (B) Tyrosine phosphorylation of CD19 was analyzed by affinity purification (AP) of CD19 from unstimulated or IgE-BCR activated cells, followed by immunoblot analysis of purified CD19 with anti-phospho-tyrosine antibodies (a-pTyr) (upper panel) and anti-CD19 antibodies as loading control. Quantification of band intensities was done as in (A). (C) Activation of the Akt kinase in anti-IgE-stimulated cells was analyzed by immunoblot analysis of cellular lysates with anti-phospho-Akt (p-Akt) antibodies (upper panel). The blot was subsequently developed with anti-Akt antibodies (lower panel) as loading control. Quantification of band intensities was done as in (A). All data are representative of at least three independent experiments.

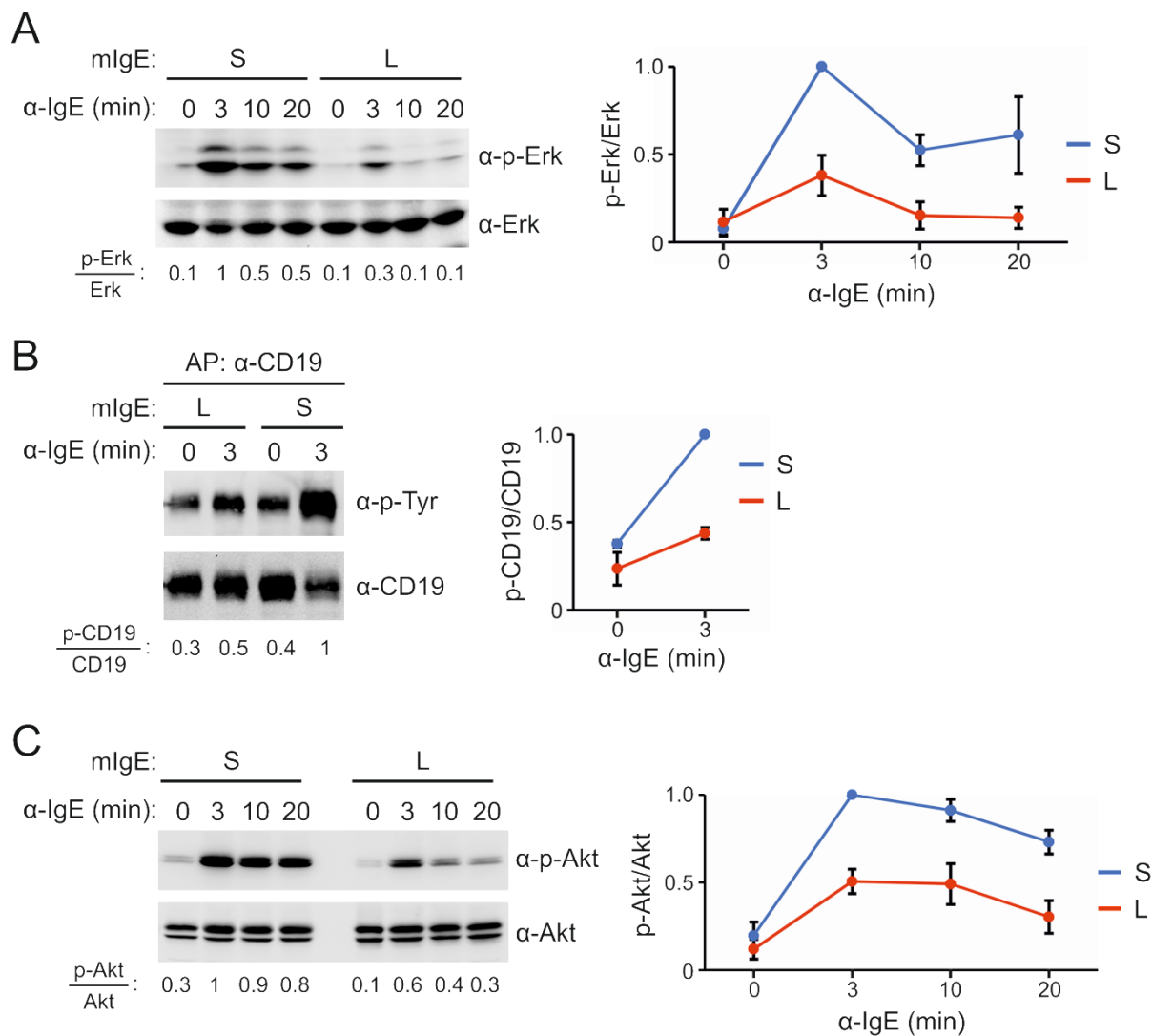


Figure 6

Figure 7. (A) DG75 cells expressing the short (mIgE S) or long (mIgE L) isoform of human mIgE were left untreated or stimulated with 20 µg/ml anti-IgE antibodies for 24 hours. As control the same cells were stimulated via their endogenous mIgM-BCRs with 20 µg/ml anti-human IgM F(ab')₂ fragments. Subsequently cells were stained with BV421-conjugated AnnexinV and 7-AAD and analyzed by flow cytometry. (B) Data analysis of experiments showing results for 24 and 48 hours of stimulation. Data are pooled from three independent experiments. Error bars represent standard deviation.

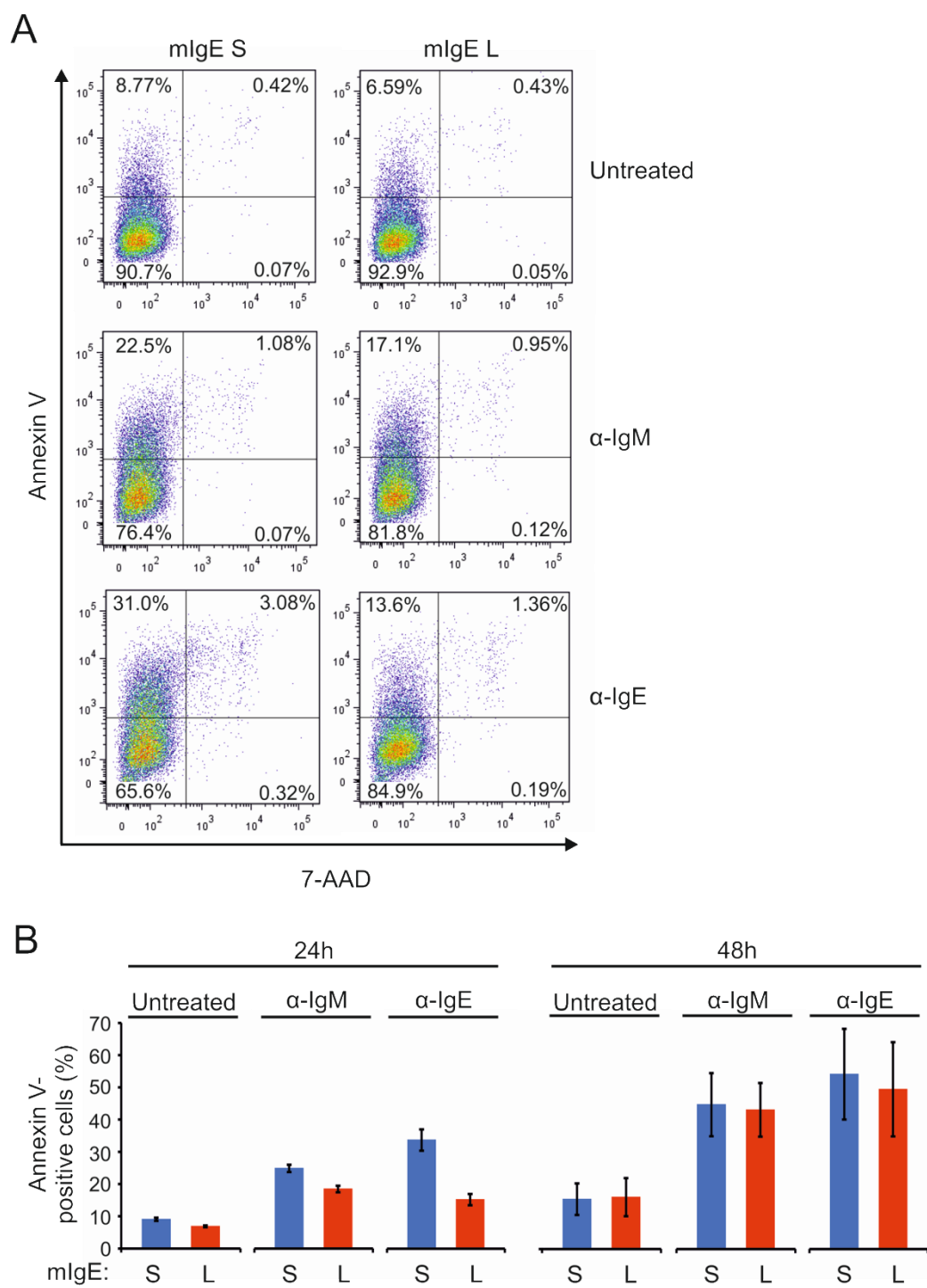


Figure 7

The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell activation by limiting B cell antigen receptor surface expression

Kanika Vanshylla, Felipe Opazo, Konrad Gronke, Jürgen Wienands and Niklas Engels

Correspondence: Dr. Niklas Engels, Georg-August-University Göttingen - Institute of Cellular & Molecular Immunology, Göttingen, Germany.

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

21-Jul-2017

Dear Dr. Engels,

Manuscript ID eji.201747196 entitled "The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell antigen receptor surface expression and signaling" which you submitted to the European Journal of Immunology has been reviewed. Both reviewers found the study very interesting, but they asked for some additional experiments to further validate the results. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referee(s) before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Marta Vuerich

On behalf of
Prof. Hans-Martin Jack

Dr. Marta Vuerich
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European Journal of Immunology
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Reviewer: 1

Comments to the Author
Manuscript by Vanshylla et al.

- This is an important, convincing and excellently performed study showing that the EMPD domain in human membrane IgE restrains IgE surface expression and signaling. The paper is written concisely and the conclusions are justified by the experiments. Referencing and scope are suitable for EJI.

- Fig. 1D. The authors show a double band for the short IgE form, the upper of which supposedly represents the fully glycosylated form. The authors should be more precise here, especially as they work in the context of ER-Golgi-Surface trafficking. N-linked glycoylations are added in the ER and replaced by O-linked glycosylation in post ER-compartments. It would add to the author's conclusions when affinity purified S and L IgE forms were digested with EndoH. Only the long IgE form should be digested by EndoH and the short form should be at least partially resistant. This experiment would be a biochemical proof that the EMPD containing IgE forms are indeed stuck in the ER.
- Discussion: It has previously been shown that ER linked Ig chains are not linked to the (pre) BCR signaling machinery, probably by an ER chaperone mediated retention mechanism (Brouns et al., JBC, 1996; Mielenz et al., JI, 2003). These references support the author's findings.
- Define AP in the abbreviations section.

Minor / at the author's discretion

p.6, l.18 „was comparable“.

p.6., l.19 „in the infected“..

p.8., l.5 „To put a finer point“...: unusual expression

p.12, l.14 „Be that as it may“...: unusual expression

Discussion: The notion that an ER-resident chaperone might trap EMPD containing transmembrane proteins is intriguing; already in 1986 and 1988 Bole, Hendershot and Kearney proposed a role for Bip in retaining nascent Ig chains in the ER.

Reviewer: 2

Comments to the Author

Essential points

1.- I think the manuscript is suitable for EJI. It is focused on the molecular regulation of human IgE, which plays a key role in Allergy. In the light of recent new findings with regard to (murine) IgE regulation from the labs of C. Allen and D. Kitamura the contribution by Vanshylla et al. definitely broadens our understanding of the many facets of antibody regulation and IgE regulation in particular

- Although the importance of the EMPD domain of human IgE isoforms has been discovered before this work mechanistically furthers our understanding by demonstrating that it is a ER retention signal. While some of the experiments on the biochemical function of EMPD have been done by the Burrone lab 21 and 11 years ago, the discrepancies are worthwhile to be presented for discussion to immunologists interested in Allergy research.

- The experiments seem to be stringent and adequate to the task at hand.

- The statistical analysis also seems to be okay and experiments have been sufficiently repeated.

- In general the conclusions drawn are warranted. However, on page 6, line 6 they correctly state that „œ ... and thus may control the production of IgE antibodies“• . In contrast to this statement they use, in the last sentence of their abstract page 3, line 14, the words: „œ“ that may control the production of anaphylactic IgE antibodies“• . I think usage of the word anaphylactic in this context is misleading, because it suggests that the EMPD would actually specifically target pathogenic IgE antibodies.

- The literature with regard to membrane IgE is adequately cited and discussed, with two notable exceptions: A, The review on general aspects of IgE and Allergy by Gould and Sutton is nearly 10 years old and better more up to date reviews would enhance the appeal of the manuscript for clinically oriented scientist, e.g. Oettgen HC J Allergy Clin Immunol 2916. B, While they cite and discuss the recent papers by Allen and Kitamura and include their own data on CD19 (Fig. 6B) they do not mention or discuss the critical point that mIgE (ectodomain) interacts with CD19 to drive the mIgE B cell into apoptosis or plasma cell differentiation. I understand that the DG75 cell line might not be able to reproduce any of these reactions, however a discussion of this might be interesting to read.

2. Specific criticism and questions

- In their work the scientists use the human DG75 cell line, which expresses mIgM. Could they elaborate, why they chose this system. In essence it is an equivalent of an early immature human B cell, or is it a memory B cell. Is it an adequate cell line for IgE expression?

- Did they think about experiments to at least partially verify their results in primary human B cells. I wonder if PBMCs from allergic persons (which should be easy to find) do have mIgE+ B cells. Alternatively one could try and isolate human B cells, culture them with IL-4 and try to transfect them with an amphotropic retroviral transduction system and the different IgE constructs and then stimulate the B cells.

- In the same line of argumentation could the authors do a PCR to test human PBMCs from allergic and

non-allergic persons to test for the presence or ratio of long vs. short mIgE. I am aware that this is not directly related to the mechanistic cell culture based topic of the paper, however if mIgE sequences can be amplified from human blood it might support the importance of the paper and the mechanism of IgE regulation postulated.

- From the data on the different signaling of long vs short mIgE it becomes clear that the mechanism must be reduced signaling by EMPD containing long mIgE, because of a reduced expression on the surface. We would suggest to try to incorporate this relation more patently in the title, because otherwise one could think that an expression independent inhibitory function of EMPD might exist: The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell antigen receptor surface expression and signaling

- Finally, we would like to ask the authors if they could provide, in addition to their biochemical data, any hint for a functional difference, with regard to either short or long mIgE. I am aware that cell lines are notoriously difficult to test, proliferation or cytokine production are limited options. But as the original paper (Batista et al JEM 1996), used human long and short mIgE transfectants of WEHI-231 to demonstrate apoptosis induction (Fig.8), it would be of importance to test if DG75 could react to anti-IgE in any way.

First Revision – authors' response

03- Oct- 2017

Manuscript ID eji.201747196

"The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell antigen receptor surface expression and signaling" by Vanshylla et al.

Point-By-Point Reply to Reviewers' Comments

We would like to thank all reviewers for taking their time to thoroughly evaluate our manuscript and the thoughtful comments and suggestions they provided. As described below, we have conducted additional experiments and revised the manuscript accordingly.

Reviewer #1:

Comments to the Author

- *This is an important, convincing and excellently performed study showing that the EMPD domain in human membrane IgE restrains IgE surface expression and signaling. The paper is written concisely and the conclusions are justified by the experiments. Referencing and scope are suitable for EJI.*

- *Fig. 1D. The authors show a double band for the short IgE form, the upper of which supposedly represents the fully glycosylated form. The authors should be more precise here, especially as they work in the context of ER-Golgi-Surface trafficking. N-linked glycosylations are added in the ER and replaced by O-linked glycosylation in post ER-compartments. It would add to the author's conclusions when affinity purified S and L IgE forms were digested with EndoH. Only the long IgE form should be digested by EndoH and the short form should be at least partially resistant. This experiment would be a biochemical proof that the EMPD containing IgE forms are indeed stuck in the ER.*

We agree with the reviewer that analysis of the glycosylation state of different mIgE isoforms can support our flow cytometric and microscopic data. Hence, we extended the biochemical experiments shown in Figure 3 (now Figure 3A) and treated short and long mIgE isoforms from either the cell surface or from total cells with EndoH (or PNGaseF as control). As anticipated by the reviewer, the cell surface-bound pool of the short isoform indeed is completely resistant to treatment with EndoH, whereas the long isoform is de-glycosylated by this enzyme and hence runs as a sharp band at a lower molecular weight. In conclusion this experiment corroborates our previous FACS- and microscopy-based observations. The new data are presented in Figure 3B.

- *Discussion: It has previously been shown that ER linked Ig chains are not linked to the (pre) BCR signaling machinery, probably by an ER chaperone mediated retention mechanism (Brouns et al., JBC, 1996; Mielenz et al., JI, 2003). These references support the author's findings.*

In case of the pre-BCR the current concept assumes that this receptor possesses some sort of autonomous, i.e. antigen-independent clustering and signaling activity. Interestingly, a similar mechanism of antigen-independent receptor-autonomous signaling activity has recently been suggested for murine mIgE-BCRs by the labs of D. Kitamura and C. Allen. However, this antigen-independent signaling activity of mouse mIgE-BCRs occurs at the plasma membrane – probably due to aggregation of murine mIgE with CD19 – and not within intracellular compartments. Thus far we have no experimental evidence that human mIgE shows antigen-independent clustering. Hence we have no reason to believe that ER-resident mIgE-BCRs would induce autonomous signaling. Nevertheless, since ER-retained long mIgE-BCRs theoretically could exist as clusters, the argument put forward by the reviewer is a valid one and hence we have addressed that point in the discussion.

- *Define AP in the abbreviations section.*

Done.

Minor / at the author's discretion

p.6, l.18 ...was comparable....

p.6., l.19 ...in the infected.....

p.8., l.5 To put a finer point.....: unusual expression

p.12, l.14 Be that as it may.....: unusual expression

Done.

Discussion: The notion that an ER-resident chaperone might trap EMPD containing transmembrane proteins is intriguing; already in 1986 and 1988 Bole, Hendershot and Kearney proposed a role for Bip in retaining nascent Ig chains in the ER.

We are aware of this mechanism and indeed we can observe an interaction between mIgE and BiP in co-immunoprecipitation experiments (data not shown). However, we could not observe a significant difference in the amount of BiP that is co-purified with the long versus the short isoform and hence we cannot nail down the mechanism of ER retention to an increased binding of the long isoform to BiP. We made a mass spectrometry-based proteomics experiment to identify binding partners for long mIgE that might give a hint to the mechanism of ER retention. However, we could not identify a promising ER-resident chaperone, which is specifically enriched for the long mIgE isoform. Since also the majority of short mIgE (or short ϵ m heavy chains) is found in intracellular compartments, this large pool of ER-resident short mIgE might blur the results. Nevertheless, we have stressed a possible role of ER quality control mechanisms in ER retention of long mIgE in more detail in the Discussion.

Reviewer #2:

Comments to the Author

Essential points

1.- I think the manuscript is suitable for EJI. It is focused on the molecular regulation of human IgE, which plays a key role in Allergy. In the light of recent new findings with regard to (murine) IgE regulation from the labs of C. Allen and D. Kitamura the contribution by Vanshylla et al. definitely broadens our understanding of the many facets of antibody regulation and IgE regulation in particular

- Although the importance of the EMPD domain of human IgE isoforms has been discovered before this work mechanistically furthers our understanding by demonstrating that it is a ER retention signal. While some of the experiments on the biochemical function of EMPD have been done by the Burrone lab 21 and 11 years ago, the discrepancies are worthwhile to be presented for discussion to immunologists interested in Allergy research.

- The experiments seem to be stringent and adequate to the task at hand.

- The statistical analysis also seems to be okay and experiments have been sufficiently repeated.
- In general the conclusions drawn are warranted. However, on page 6, line 6 they correctly state that “..... and thus may control the production of IgE antibodies”. In contrast to this statement they use, in the last sentence of their abstract page 3, line 14, the words: “... that may control the production of anaphylactic IgE antibodies”. I think usage of the word anaphylactic in this context is misleading, because it suggests that the EMPD would actually specifically target pathogenic IgE antibodies.

Thank you for this comment. Indeed the original phrasing might be misinterpreted. We have edited this sentence, which now reads “... that may control the production of potentially anaphylactic IgE antibodies”.

- The literature with regard to membrane IgE is adequately cited and discussed, with two notable exceptions: A, The review on general aspects of IgE and Allergy by Gould and Sutton is nearly 10 years old and better more up to date reviews would enhance the appeal of the manuscript for clinically oriented scientist, e.g. Oettgen HC J Allergy Clin Immunol 2916. B, While they cite and discuss the recent papers by Allen and Kitamura and include their own data on CD19 (Fig. 6B) they do not mention or discuss the critical point that mIgE (ectodomain) interacts with CD19 to drive the mIgE B cell into apoptosis or plasma cell differentiation. I understand that the DG75 cell line might not be able to reproduce any of these reactions, however a discussion of this might be interesting to read.

Ad A) In the revised manuscript we now also cite the review article by HC Oettgen (Reference 5).
Ad B) The reason why we did not elaborate on the presumed role of CD19 for autonomous signaling of mIgE-BCRs is that it seems to be specific for murine mIgE and hence does not add much to the understanding of our data. First, we did not observe clear signs of mIgE-autonomous (i.e. crosslinking-independent) signaling like elevated basal Ca²⁺ levels, increased basal phospho-Erk, etc. Second, we have tried to reproduce the interaction between human mIgE and CD19 in human B cells using the protocol described by the group of D. Kitamura. However, we could not detect an interaction between the human orthologues. Also our proteomics approach to identify mIgE interaction partners did not show an interaction of mIgE with CD19 (see above, data not shown). We are aware that the inability to detect a certain protein-protein interaction does not disprove or refute anything. However, an amino acid sequence comparison between the region of mouse ϵ m that was reported to interact with CD19 with that of human ϵ m reveals that there is only limited sequence similarity (roughly 50%). This lack of sequence conservation might explain why we do not see an association between human mIgE and CD19.

2. Specific criticism and questions

- In their work the scientists use the human DG75 cell line, which expresses mIgM. Could they elaborate, why they chose this system. In essence it is an equivalent of an early immature human B cell, or is it a memory B cell. Is it an adequate cell line for IgE expression?

Previous studies on human mIgE such as those by Batista et al. (Ref. 7) and Pogganiella et al. (Ref. 26) were usually done in mouse B cell lines. Given the low overall sequence similarity between mouse and human ϵ heavy chains, we figured that a human B cell line represents a more suitable model system to study human mIgE.

The DG75 cell line was established from a Burkitt lymphoma and thus most likely represents a germinal center stage. It has been used before to study immunoglobulin isotype-specific functions, especially BCR signaling (Ref. 14). Furthermore, our key observation – limited surface expression of the long mIgE isoform – is also observed in primary B cells (see Fig. 1E) as well as in additional B cell lines of mouse and human origin like WEHI-231, J558L and Ramos (data not shown). All things considered, we think that the DG75 cell line is a valid model system to study immunoglobulin/BCR isotypes.

- Did they think about experiments to at least partially verify their results in primary human B cells. I wonder if PBMCs from allergic persons (which should be easy to find) do have mIgE+ B cells. Alternatively one could try and isolate human B cells, culture them with IL-4 and try to transfect them with an amphotropic retroviral transduction system and the different IgE constructs and then stimulate the B cells.

We appreciate this comment since it gave us the chance to close a small gap in our line of argumentation. However, we had to slightly modify the suggested experiment. In the original discussion we elaborated on the phenotype of a mouse strain, in which the (human) EMPD was knocked into the mouse ϵ heavy chain locus, creating a mouse/human (long) ϵ m chimera. This mouse line (Ref. 16) as well as another recently reported mouse line with a similar EMPD knock-in (Ref. 27) have severely reduced serum IgE titers, which may be explained by the fact that the chimeric long mIgE is retained in the ER of primary mouse B cells as it is in the DG75 cell line. To test this notion we have used primary mouse B cells instead of human B cells. Indeed the experiments with primary mouse B cells recapitulate our observations made in DG75 cells. Hence, it seems reasonable to assume that the EMPD limits IgE antibody production *in vivo*.

- In the same line of argumentation could the authors do a PCR to test human PBMCs from allergic and non-allergic persons to test for the presence or ratio of long vs. short mIgE. I am aware that this is not directly related to the mechanistic cell culture based topic of the paper, however if mIgE sequences can be amplified from human blood it might support the importance of the paper and the mechanism of IgE regulation postulated.

We agree with the reviewer that this is an interesting experiment that might provide insight into the development of allergic reactions. However, we also agree that this experiment is not directly related to the topic of our manuscript. Furthermore, such an approach is technically demanding, since it should ideally be done using single cell PCRs that take into account the developmental stage of the analyzed cells (memory, plasmablast, germinal center...). Since mIgE-positive B cells are notorious for their scarcity (especially in blood) and difficulty to identify, this experiment unfortunately seems to be beyond the scope of this study.

- From the data on the different signaling of long vs short mIgE it becomes clear that the mechanism must be reduced signaling by EMPD containing long mIgE, because of a reduced expression on the surface. We would suggest to try to incooperate this relation more patently in the title, because otherwise one could think that an expression independent inhibitory function of EMPD might exist:

The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell antigen receptor surface expression and signaling

Again we would like to thank the reviewer for the thoughtful comment. To avoid possible misinterpretations, we have adjusted the title, which now reads "The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell activation by limiting B cell antigen receptor surface expression".

- Finally, we would like to ask the authors if they could provide, in addition to their biochemical data, any hint for a functional difference, with regard to either short or long mIgE. I am aware that cell lines are notoriously difficult to test, proliferation or cytokine production are limited options. But as the original paper (Batista et al JEM 1996), used human long and short mIgE transfectants of WEHI-231 to demonstrate apoptosis induction (Fig.8), it would be of importance to test if DG75 could react to anti-IgE in any way.

We agree that testing the capacity of mIgE-BCRs to induce apoptosis (or activation-induced cell death) is interesting and important, especially since mIgE-BCR-induced apoptosis has recently been suggested as a mechanism to limit IgE antibody production and memory in the mouse. Therefore, we have stimulated short and long mIgE-BCRs in our DG75 transfectants for 24 and 48 hours and tested apoptosis induction by staining with AnnexinV and 7-AAD. The results show that in DG75 cells both long and short mIgE-BCRs induce apoptosis. Whereas after 24 hours the short mIgE-BCR is somewhat more efficient, which is in line with its increased signaling capacity, the difference between the two isoforms is no longer seen after 48 hours of stimulation. As control we stimulated the endogenous mIgM-BCR of the transfectants, which yielded in similar percentages of apoptotic cells. In the Batista paper (JEM, 1996) crosslinking of long mIgE had no detectable effect on cell proliferation. However, back then the analysis was done only after 24 hours and growth inhibition was clearly dependent on the dose of the anti-IgE antiserum. Since we have used a different batch of anti-IgE antibodies (that most likely has a different average affinity and crosslinking ability), a different cellular background and polyclonal transfectants instead of monoclonal lines, it is difficult to compare our results with those of the Batista paper. At least we can conclude that human mIgE-BCRs have the capacity to induce apoptosis in susceptible cell lines such as WEHI-231 and DG75. However, whether this happens *in vivo* in primary human B cells that have undergone immunoglobulin class-switching to IgE remains to be investigated.

Second Editorial Decision

19-Oct-2017

Dear Dr. Engels,

It is a pleasure to provisionally accept your manuscript entitled "The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell activation by limiting B cell antigen receptor surface expression" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Marta Vuerich

on behalf of
Prof. Hans-Martin Jäck

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