

Recombinant IgE antibody engineering to target EGFR

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Abstract Monoclonal antibodies have become a mainstay for the targeted treatment of cancer today. Some of the most successful targets of monoclonal antibodies are constituted by the epidermal growth factor receptor family spearheaded by the epidermal growth factor receptor (EGFR). Prompted by studies indicating that IgE compared to IgG may harness alternate effector functions to eradicate malignant cells, we addressed the establishment, engineering, and the potential tumoricidal effects of recombinant anti-EGFR IgE. Therefore, two different therapeutic EGFR-specific antibodies, 225 and 425, were chosen for re-cloning into different chimeric IgE and IgG formats and produced in human cells. Simultaneous antibody binding to the sEGFR demonstrated accessibility of both epitopes for

recombinant IgE. Proliferation and cytotoxicity assays demonstrated signal blocking and effector mediating capability of IgE isotypes. Pronounced degranulation in the presence of sEGFR upon activation exclusively with two IgE antibodies verified the epitope proximity and provides evidence that tumor-targeting by anti-EGFR IgE is safe with regard to soluble target structures. Degranulation mediated by tumor cells expressing EGFR could be demonstrated for singular and combined IgE antibodies; however, use of two IgE specificities was not superior to use of one IgE alone. The data suggest that the surface distribution of EGFR is optimally suited to mount a robust effector cell trigger and corroborate the potential and specificity of the IgE/IgE receptor network to react to xenobiotic or pathogenic patterns for targeting malignancies.

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The EGF receptor family

In the early 1980s, when the avian erythroblastosis tumor virus was found to encode an aberrant form of the human epidermal growth factor receptor (EGFR) [1], the ERBB receptor tyrosine kinases (RTK) were implicated in cancer [2]. Consequently, their signal transduction pathways involved in the regulation of cellular proliferation were studied in detail, and aberrations in tumor cells were postulated causally involved in malignant transformation.

The EGFR group rapidly has expanded to four members (EGFR/ERBB1–ERBB4, also known as HER1–4) with 13 polypeptide extracellular ligands which contain a conserved epidermal growth factor (EGF) domain [3]. The

ERBB receptors are glycosylated transmembrane proteins composed of a large extracellular ligand-binding domain, which has four subdomains (I–IV), followed by a transmembrane domain, a small intracellular juxtamembrane domain, preceding the kinase domain, and a C-terminal tail, on which the docking sites for phosphotyrosine-binding effector molecules are found.

The basic functional unit in ERBB signaling is the receptor dimer to which each partner contributes unique features. There is evidence that the ERBB receptors exist in a pre-dimerized state [4] and ligand binding forms a 2:2 ligand to receptor configuration [5]. An important issue of the ERBB network is the non-autonomy of two receptors, the ligand-less ERBB2 (also known as HER2), and the almost kinase inactive ERBB3. Despite the lack of autonomy, both ERBB2 and ERBB3 form heterodimeric complexes with the other ERBB receptors that are capable of generating potent cellular signals. Furthermore, ERBB2 containing heterodimers are characterized by a higher affinity and broader specificity for various ligands than other receptor heterodimers. Additionally, ERBB2-containing heterodimers undergo slow endocytosis, and recycle more rapidly back to the surface [6, 7].

The EGFR constitutes the prototype component of the ERBB receptor signaling network, which controls multiple cellular activities through binding of a large group of partly redundant ligand growth factors that are translated into activation of multiple transcription factors [3]. It forms homodimers as well as three functional heterodimers. This dimerization induces receptor auto-phosphorylation and subsequently recruits a number of adaptor proteins such as GRB2 and SH2 proteins to the phosphorylated receptor. By recruiting the ubiquitin ligase CBL, the EGFR gets ubiquitinated at its C-terminus [8]. The ubiquitin tags then target the receptor to lysosomes for degradation. Deubiquitinating enzymes, however, can target the EGFR to the recycling pathway. Ubiquitination and lysosomal trafficking of ERB2 are far less efficient than that of EGFR [7, 9], a fact that results in faster disappearance of EGFR from the surface.

GRB2, however, can initiate downstream signaling which is responsible for the recruitment of Ras which activates kinase cascades such as the RAF-MEK and PI3K-PDK-AKT pathway [10]. After EGF stimulation, the cytoplasmic kinase c-SRC phosphorylates Tyr-845 and thereby hyperactivated the EGFR [11, 12] and other signaling molecules such as JAK2 and STAT3 [13], SHC and components of the cytoskeleton and the endocytic machinery [14]. Activation of these pathways results in proliferation, differentiation, and/or oncogenesis of epithelial cells, neuronal cells, and fibroblasts [15]. Enhanced EGFR expression and activation thus has been found in a variety of human tumor cells. High levels were correlated with a poor response to treatment, disease progression, and

shortened survival periods, hence an unfavorable prognosis and a high incidence of metastasis [16]. Nevertheless, a variety of factors [17] including but not limited to EGFR copy number, mutation status of EGFR and KRAS, ubiquitination and cellular localization of EGFR, and increased epithelial-mesenchymal transition are supposed to support tumor resistance rendering the EGFR interaction landscape broad and multifaceted.

EGFR as target for anti-EGFR therapeutics

Hence, EGFR represents a key target for the development of therapeutic approaches including all types of potential drugs. These include a variety of different affinity molecules such as antibodies, peptide [18] and nucleic acid [19] aptamers, low molecular weight compounds such as specific and pan-specific receptor tyrosine kinase inhibitors (gefitinib, erlotinib, PKI166, canertinib, tyrphostins, etc.), and some natural compounds. Additionally, other nucleic acid-based approaches using antisense, ribozyme [20], or siRNA technologies and combinations thereof are pursued. Albeit this plethora of affinity molecules and additional concepts such as B cell epitope vaccination [21–23] are in the pipeline, approved anti-EGFR therapeutics so far are limited to two monoclonal antibodies (cetuximab, panitumumab) and tyrosine kinase inhibitors (gefitinib, erlotinib).

The concept of applying blocking mAbs to the EGFR as an approach in cancer therapy was first developed by Mendelsohn in the 1980s [24]. His group generated two blocking anti-EGFR mAbs—mAb 528 and 225—which inhibited the *in vitro* and *in vivo* growth of human cancer cell lines that express EGFR [25]. More than 20 years later, the monoclonal antibodies cetuximab and panitumumab obtained approval for EGFR-targeted immunotherapy.

The first approved anti-EGFR antibody cetuximab is a chimeric murine/human monoclonal antibody of the IgG1 k isotype derived from the above-mentioned murine mAb 225. After cetuximab binding, the EGFR rapidly disappears by internalization and trogocytosis [26]. Hence, interference with signal transduction is considered to be the primary anti-tumor mechanism and the fundament for synergy with chemotherapy rather than via immunotherapy mechanisms. Unlike most mAbs that are currently approved for clinical use, cetuximab is manufactured in murine plasmacytoma cells which adds alpha-1,3-galactose units eliciting immune responses and IgE mediated anaphylaxis in certain patients [27].

Panitumumab in contrast is a fully human, xeno-mouse derived IgG2 k antibody [28]. Although less immune responses are reported for the human molecule, both approved antibodies provoke neutralizing antibodies in a number of patients. Both of them compete with

endogenous ligands like EGF and TGF- α [29]. Cetuximab has been approved for use in combination with irinotecan in advanced-stage EGFR-expressing metastatic colorectal carcinoma and also for patients with advanced squamous cell carcinoma of the head and neck in 2004 and 2006 (US), respectively [30]. Panitumumab has been approved for monotherapy of metastatic colorectal carcinoma in 2006 and 2007 (EU), respectively.

Other anti-EGFR antibodies under development include but are not limited to zalutumumab, nimotuzumab, and matuzumab. Zalutumumab is a fully human antibody [31], whereas nimotuzumab and matuzumab are humanized antibodies originating from hybridoma lines. Like cetuximab, the humanized matuzumab or EMD 72000 is derived from a murine hybridoma—here the 425 antibody—which hinders binding of ligands and thus EGFR activation [32] and inhibits tumor growth in mouse models [33]. Development for immunotherapy as humanized version in colorectal carcinoma has been discontinued, but other clinical trials on combination therapy are ongoing [34, 35].

Nimotuzumab is a humanized version of the hR3 antibody developed in Havana, Cuba [36, 37], which is used in cancer therapy outside of the US and EU. Its reduced side effects are attributed to its lower affinity resulting in improved pharmacokinetics with tumor-specific enrichment without significant binding to EGFR-expressing epithelial cells in the skin.

Another and a particular antibody under development is the humanized antibody 806 which specifically targets the EGFR deletion variant de2-7 EGFR [38]. This antibody is currently assessed in a phase 1 study in subjects with advanced solid tumor types.

With respect to their molecular interaction mechanisms, some antibodies such as the antibody 225 recognize epitopes within the ligand-binding site and therefore compete with the natural ligand. Panitumumab as well as nimotuzumab do also compete with the ligand and thus are mechanistically comparable. Others such as the antibody 425 seem to recognize variant epitopes preventing structural rearrangements of the receptor thus indirectly hampering ligand binding and receptor activation [39].

For studies on the therapeutic potential of variant human isotypes for targeting tumor cells, we focussed on the EGFR and corresponding anti-EGFR antibodies, a scenario which is orchestrated by the large variety of players interacting with each other. With especially more than one type of anti-body and some structural information being available, the impact of epitope localization becomes addressable. Furthermore, the effector mechanisms of EGFR-targeting antibodies such as cetuximab which are based on Fc receptor and complement interactions seem to be poorer as compared to other antibodies such as rituximab (Roche) and trastuzumab (Roche), for which

antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) constitute essential mechanisms [40]. Another very recent finding even suggests detrimental tumor-promoting effects of the IgG format of cetuximab being dependent on its Fc fragment [41]. In this regard, studying the benefit of isotype variants of EGFR-specific antibodies different from IgG is highly relevant and mechanistically challenging.

The antibodies chosen for comparative isotype assessment are the clones 225 and the 425, antecessors of cetuximab and matuzumab which apparently differ with regard to their epitopes and therefore their mechanistic characteristics. In this setting, individual, additive, and synergistical effects of recombinant IgE thus might become better discernible.

Engineering of recombinant IgE: formats, production, and activity

Recombinant antibody technologies became available in the early to mid-1980s [42–44], and most attention has since then been given to the IgG subclasses. In contrast, little attention was paid to other isotypes, a fact that might be attributed to mere historic and regulatory reasons as well as the need for more sophisticated recombinant antibody technologies. As a consequence, all antibodies approved so far are of the IgG isotype. For a long time, the field of alternative isotypes including the lowest abundance isotype IgE languished. The resulting scarcity of specifically designed IgE antibodies has thus far prohibited detailed analyses of their characteristics in pathophysiology as well as their molecular interplay with Fc receptors and effector cells. As early as in 1987, murine IgE was produced in recombinant form [45]. Recently, IgE entered the field of human isotypes being available in recombinant form. Neuberger and colleagues were the first to establish the well-known anti-NP IgE by transfecting the human/mouse chimeric epsilon chain while retaining the murine light chain. Chimerization of murine antibodies still is relatively time-consuming; nonetheless, the re-cloning of murine hybridomas to chimeric IgE has been reported [46, 47]. With the establishment of combinatorial approaches, human-binding moieties became accessible and fully human isotypes including IgE exemplary were established using human antigen-binding regions [48]. The characteristics of this IgE, however, were not translated to targeting approaches and remained elusive.

Recombinant production of allergen-specific, fully human IgE was first demonstrated by our group in a recent approach [49] followed by several groups applying similar approaches [50–52]. The use of murine and human antigen-binding moieties and their conversion into human or

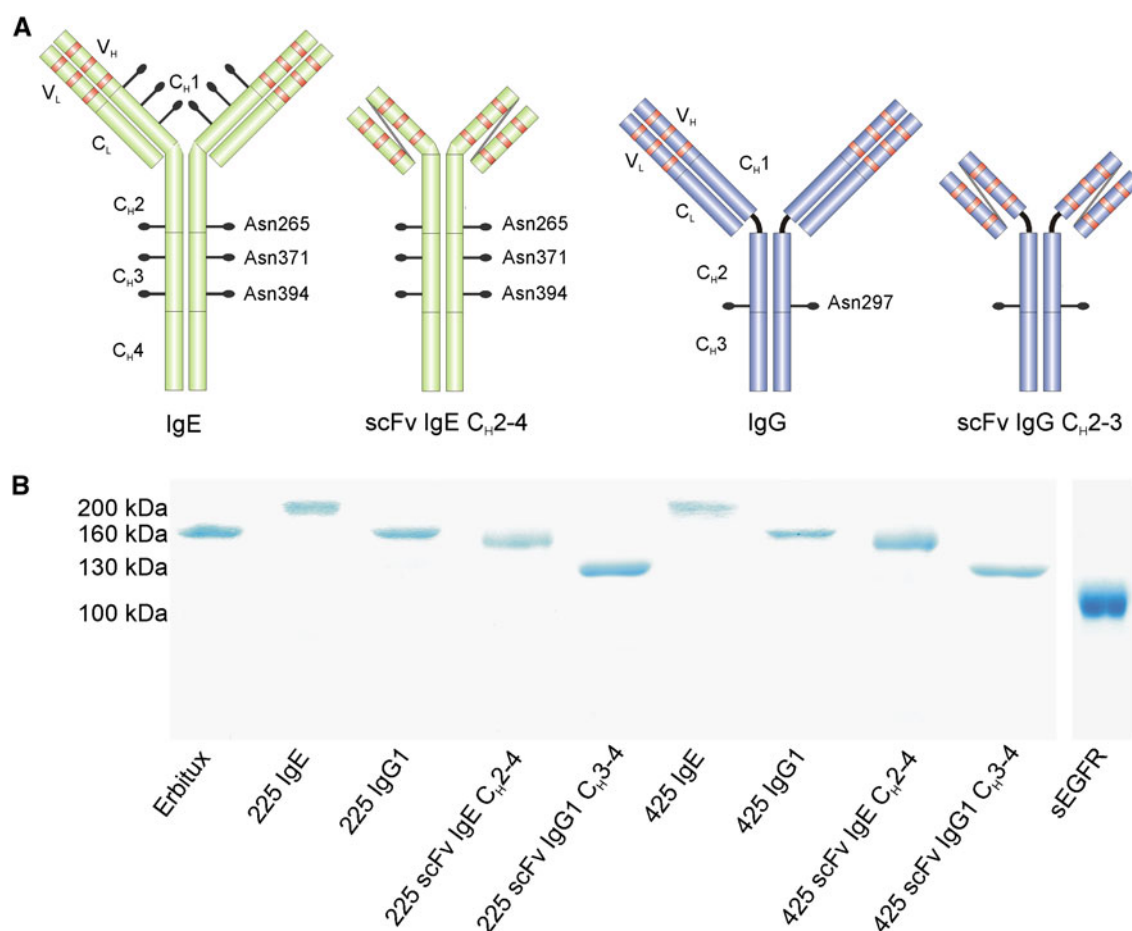


Fig. 1 Schematic representation of the recombinant immunoglobulins. **a** Heterotetrameric IgE and IgG and homodimeric scFv-IgE C_H2-4 and scFv-IgG C_H2-3 derivatives. The constant regions of the heavy and light chains and the variable regions are colored, the linker in the scFv darker. Indicated are the different domains of the

antibodies, the complementarity determining regions (CDR), and the Fc glycosylation sites. **b** SDS-PAGE analysis of purified recombinant IgE and IgG formats of EGFR-specific antibodies 225 and 425. In parallel, the parental product Erbitux and sEGFR are shown

chimeric IgE nowadays is relatively established. Nevertheless, each laboratory active in the field has developed individual vector systems using different expression formats, hosts, and purification strategies with unique characteristics.

For recombinant production of anti-EGFR recombinant antibodies (Fig. 1), we used vector systems initially established by us for expression of different immunoglobulin isotypes as miniaturized scFv-based and homodimeric constructs or heterotetrameric authentic immunoglobulins [49]. The homodimeric antibody formats lack the CH1 and the CL domains resulting in a reduced molecular weight and do not require light chain assembly with heavy chains. Although quality control in the ER might be less stringent due to absence of chaperone-binding sites, the yields of such formats is up to 10–20 times higher compared to the heterotetrameric format, rendering these interesting for initial proof of concept analyses and binding studies [53]. Such IgE formats can even be further minimized but receptor binding requires CH3-4 with CH2

modulating interaction [54]; hence, the scFv-CH2-4 most likely is the smallest suitable construct possible.

Generally, the secretion efficiencies obtained for recombinant IgE without any further optimization are highly depending on the individual antibody with the IgE having lower concentration in cellular supernatants compared to the IgG isotype.

As expression host, we stably transfected HEK-293 cells representing a widely established and robust human cell line which is easily adaptable to different culture conditions and provides a human glycosylation that circumvents any interference from carbohydrate-based reactivities. Differentially formatted 225- and 425-derived IgE and IgG1 antibodies were purified from the cell culture supernatants by affinity chromatography using matrices specific either for a C-terminal histidine-tag or for the kappa constant light chain. Soluble antigen for analyses of the 225 and 425 antibodies is readily available as an EGFR splice variant (sEGFR) comprising the extracellular ligand-binding

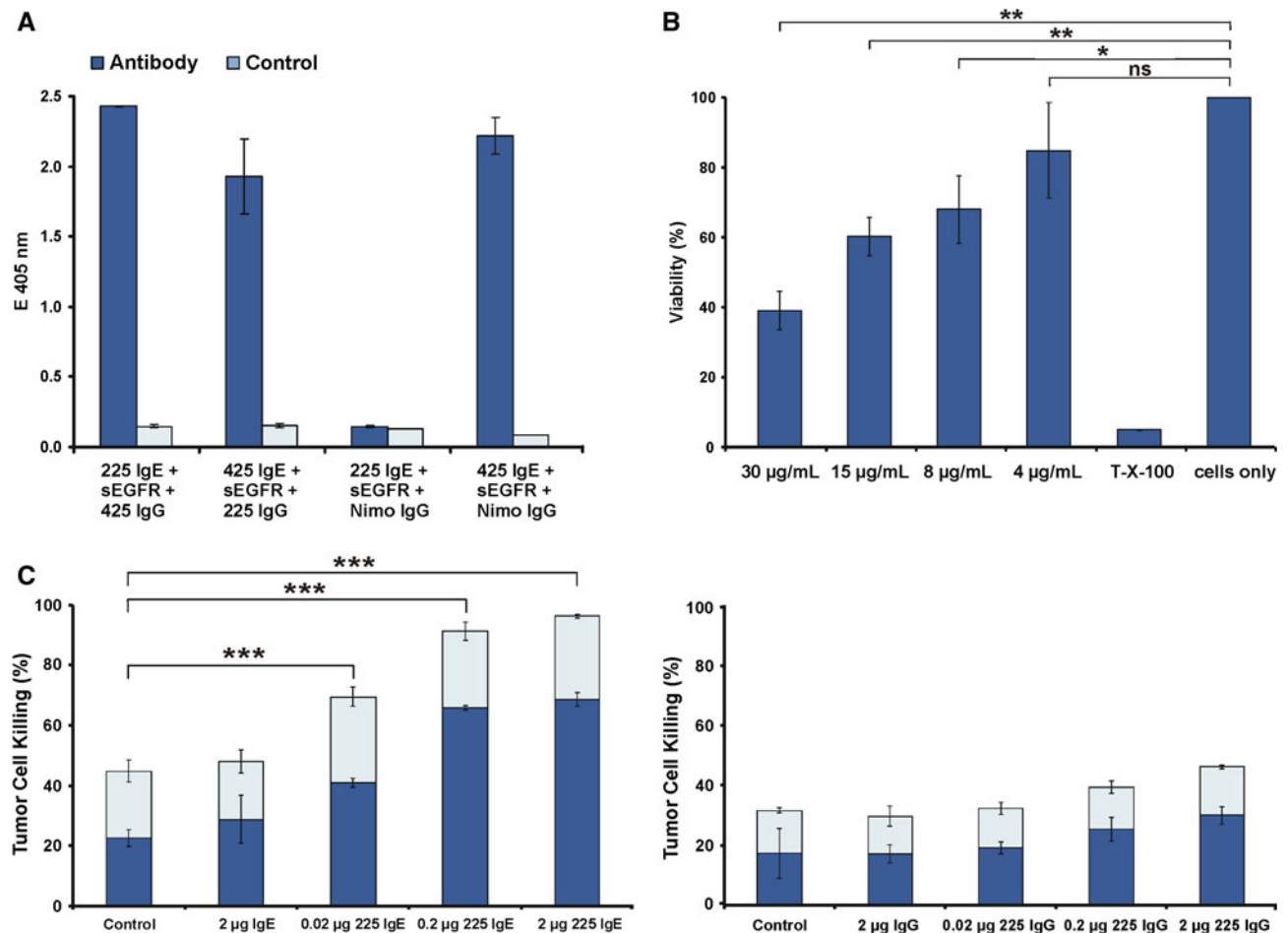


Fig. 2 Analyses of recombinant IgE. **a** Sandwich-based assessment of the epitope architecture and accessibility by three types of IgE and IgG antibodies, 225, 425, and nimotuzumab, the latter as IgG only. **b** MTT assay using the 425 IgE antibody. **c** Three-color assay for the

assessment of tumor cell killing by the 225 IgE (left) and 225 IgG (right) showing the ratio of phagocytosis (bright blue) versus cytotoxicity (dark blue)

domain. This sEGFR is naturally secreted by A431NS epidermoid carcinoma cells and can be affinity-purified from the culture supernatant [55].

Even though other expression strategies and hosts might provide different features or advantages, our modular vector system and purification approaches represent a straightforward strategy for the generation of a variety of antibody isotypes from different species.

The availability of sEGFR and different isotypes enabled us to assess the characteristics of the recombinant immunoglobulins and to dissect the epitope architecture in sandwich-based assays (Fig. 2a). These analyses provided evidence that 225 and 425 antibodies can bind simultaneously to EGFR suggesting independent IgE epitopes, whereas binding of 225, not 425 was blocked by nimotuzumab indicating overlapping epitopes of 225 and nimotuzumab.

These data are supported by recent functional [56] and also structural analyses. It has been recognized from recent crystallographic analyses that the 225 and the 425

antibodies in fact do recognize spatially distinct, not overlapping structures on the EGFR domain III [39]. More precisely, 225 binds to a conformational epitope which mainly consists of positively charged and polar uncharged side chains via hydrogen or salt bonds [57] sharing an overlapping epitope with the ligands, respectively. The binding site of 425 is located on EGFR domain III in close proximity, but not overlapping with the 225 epitope [39]. Panitumumab, however, recognizes identical residues in domain III as 225 [58], and also nimotuzumab was shown to have overlapping epitopes with both 225 and panitumumab [59].

For additional quality control of the recombinant antibodies and to confirm the interaction with the recombinant IgE and IgG1 with the ligand-binding domain of the high affinity IgE receptor and the high affinity IgG receptor CD64, respectively, soluble receptor-IgY chimeras (FcεRI-IgY Fc [60] and CD64-IgY Fc) mimicking the natural membrane-bound receptors were used in ELISA (Plum et al.,

manuscript submitted). After capturing both isotypes with immobilized sEGFR and subsequent incubation with Fc ϵ RI-IgY Fc or CD64-IgY Fc, the interaction with the particular Fc receptors could be documented. These data on functionality were additionally backed with SPR analyses. The comparable K_D values corroborate that recombinant IgE generally have molecular characteristics and functionality corresponding to the parental antibodies.

Targeting of the EGFR by IgE

The mechanism of anti-tumor activity of individual anti-ERBB receptor mAbs is not yet entirely understood. In vitro and xenograft models, cetuximab administration induces receptor internalization, blocks phosphorylation of the intracellular tyrosine kinase domain, and induces cell cycle arrest in G1 and finally, apoptosis. These findings suggest that an apoptotic or cytostatic signal is transduced through the receptor [61]. As stated above, for the therapeutic antibodies, trastuzumab and rituximab results obtained in mouse knockout models for Fc receptors strongly suggest that most of the tumoricidal activity is due

to the recruitment of natural killer cells and efficient ADCC [40]. CDC as important effector function of rituximab and campath apparently cannot be mediated via cetuximab.

Against this background, we showed that recombinant IgE targeting of the EGFR mimicks the blockade of EGFR signaling by therapeutic IgG isotypes and clearly exerts anti-proliferative effects in an Fc domain independent manner (Fig. 2b).

In contrast to the comparable blocking effects, the Fc-dependent effector mechanisms of EGFR-specific human recombinant IgE and IgG1 antibodies exhibit clear cut differences in established three-color cytometric assays [62, 63] (Fig. 2c). Using purified monocytes as effector cells, simultaneous analyses of phagocytosis and cytotoxicity exerted on A431 tumor cells revealed striking differences when administering IgE or IgG1. While phagocytosis remained nearly identical, cytotoxicity and thus overall tumor cell killing increased up to 95 %. Similar results were obtained for the 225 IgE using the human leukemic monocyte lymphoma cell line U937 cells as effector cells (Plum et al., manuscript submitted).

To assess the relevance of the epitope distribution and architecture for inducing degranulation, RBL-SX38

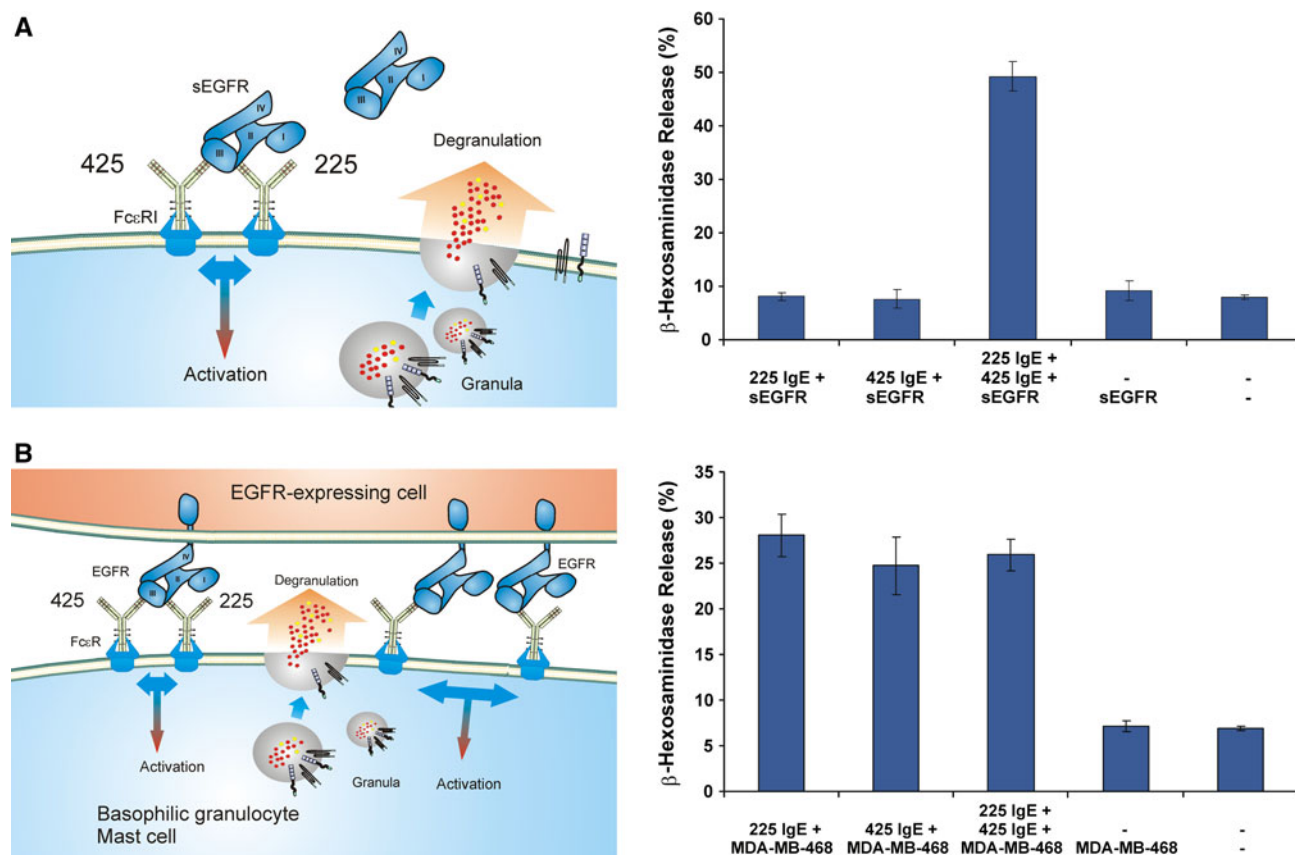


Fig. 3 Impact of epitope architecture and availability of the EGFR on the degranulation of effector cells. Soluble EGFR (a) and surface-thethered EGFR (b) were applied to effector cells loaded with 225

IgE, 425 IgE, or both IgE. Release indicates the EGFR capability of cross-linking the Fc ϵ RI-bound IgE

effector cells were loaded with 225 IgE, 425 IgE, or a combination of both and stimulated with either soluble EGFR or EGFR tethered on the cellular surface (Fig. 3). Interestingly, sEGFR together with any of the singular IgE antibodies did not provoke any activation, whereas for the biconal combination of IgE, pronounced activation was observed (Fig. 3a).

These distinct findings suggest firstly that, indeed, 225 and 425 have independent but nevertheless for large IgE sterically available epitopes in an architecture that enables efficient cross-linking of the receptor. Secondly, these data imply that EGFR can be present as monomeric molecule in solution and soluble antigen in the bloodstream would not induce anaphylactic reactions. This is of particular relevance since EGFR can be shed from cell membranes *in vivo*.

In contrast, MDA-MB-468 cells overexpressing EGFR provide antigen organized within the cellular matrix. This EGFR membrane network evidently represents an oligomerized state that is able to efficiently override the activation threshold and to induce pronounced degranulation of effector cells (Fig. 3b). Interestingly, the combination of both IgE did not result in any additive or synergistic effect, at least in the double experimental setting used here. The latter effect implies that loading of effector cells with a unique specificity is as efficient as providing two antibodies and thus the possibility to react to the same antigen. Against the background of the highly specific and efficient combination of 225 and 425 IgE, this finding suggests that a surface clustering of EGFR generates a target cell-associated molecular pattern which has been designated TAMP recently [64] that serves as activation scaffold for IgE-harnessed effector cells.

Concluding remarks

We have produced anti-EGFR-derived recombinant IgE and IgG1 in order to gain insights into the mechanisms which an IgE version of therapeutic antibodies could exploit in tumor cell targeting. Engineered recombinant IgE formats were fully functional regarding simultaneous antigen and Fc ϵ RI-binding and decreased tumor cell viability confirming that receptor blockade in general is not affected by its isotype. Whether any differences regarding receptor internalization and turnover rates of EGFR may exist and whether Fc receptor binding translates into differential activation of effector cells needs to be further investigated.

Soluble EGFR as representative of secreted splice variants or EGFR shed by proteolysis or trogocytosis *in vivo* was not sufficient for cellular activation for a singular EGFR-specific IgE. In contrast, two antibodies or oligomeric EGFR clustered on tumor cell surfaces or

EGFR-coated microspheres (not shown) efficiently activated the degranulation machinery, a finding which may help to elucidate the interplay of IgE-loaded effector cells with its cognate target structures. Hence, EGFR-specific IgE-based interventions appear to be safe with respect to shed antigen in the circulation. Further evidence was provided very recently pursuing a similar approach on the basis of an alternative IgE [65].

Of special interest in the future will be the assessment of EGFR density and cluster size needed for recruitment of effector cells and the induction of downstream effects. In our studies, IgE-harnessed effector cells were able to eradicate EGFR-expressing tumor cell lines and showed significantly variant ratios of phagocytosis and cytotoxicity compared to IgG1 for different cell lines (data not shown). The impact of target cell morphology or antigen density which might differentially sensitize target cells to cytotoxic mechanisms remains to be determined, and the information should be validated by other IgE-based targeting approaches. Alternative approaches of using recombinant IgE target other molecules of high importance, such as the folate receptor and HER-2 [62, 66] which were selected according to stringent criteria, such as high level expression, specific expression on tumor cells, no crosslinking by soluble forms, etc. and are complemented by Th2 biased vaccination strategies [67].

Hopefully, all these approaches will converge and contribute to broaden our understanding of differential functions of human immunoglobulin isotypes and to elucidate and retrieve the potential which appears within reach for therapeutic IgE antibodies.

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Conflict of interest The authors declare that they have no conflict of interest.

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