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Abstract

In combination with chemotherapy or radiation, passive immunotherapy with monoclonal antibodies is state of the art in cancer therapy. For this purpose, two properties of antibodies are harnessed: i) via the Fab fragment they bind a specific tumour antigen and ii) via the Fc portion they recruit effector cells and activate the complement system. One of these antibodies is trastuzumab (Herceptin®), a growth-inhibitory humanized monoclonal IgG1 antibody recognizing the tumour antigen HER-2, which is overexpressed in 30% of human breast cancers. Interestingly, all antibodies applied for passive immunotherapy are so far exclusively of the IgG subclass.

In contrast, antibodies of the IgE subclass are best-known for their detrimental function in type I hypersensitivity. It is little-known that IgE has anti-tumour capacity which could be exploited for immunotherapy of cancer. Thus, the aim of this doctoral thesis was to examine alternative strategies for cancer treatment based on IgE antibodies, and to compare their efficacy with that of IgG.

The oral immunization route is well suited for the induction of a Th2 immunity including high affine IgE responses to administered antigens. Therefore, the establishment of an IgE-dependent food allergy model in mice is described, which we applied also for our cancer studies. When mice were fed with different concentrations of ovalbumin under concomitant anti-acid medication, an antigen-specific IgE induction in a Th2 environment could be achieved. This oral vaccination regimen was also used for feeding HER-2 mimotopes, i.e. epitope-mimics of the anti-HER-2 IgG antibody trastuzumab. Indeed, these mimotopes induced IgE antibodies recognizing the tumour antigen which were able to bind HER-2 overexpressing breast cancer cells and led to tumour cell lysis. Complementary to this active immunotherapeutic approach a trastuzumab-like IgE antibody for passive immunotherapy was constructed. We could show that this trastuzumab IgE exhibited the same specificity as the

original trastuzumab IgG. Moreover, a three-colour flow cytometric assay indicated that even though both antibodies were able to induce tumour cell killing, they used different mechanisms: trastuzumab IgG acted via antibody-dependent cell-mediated phagocytosis (ADCP), whereas trastuzumab IgE elicited mostly antibody-dependent cell-mediated cytotoxicity (ADCC).

Based on the presented data we conclude that tumour-specific IgE, induced actively or applied in a passive manner, represents a potent alternative to IgG-based immunotherapies against cancer.

Zusammenfassung

Ergänzend zu Chemotherapie und Strahlentherapie ist die passive Immuntherapie mit monoklonalen Antikörpern eine moderne Behandlungsmethode in der Krebstherapie. Zwei Eigenschaften von Antikörpern werden dabei genutzt: i) über das Fab Fragment binden sie spezifisch Tumorantigene und ii) über den Fc Teil rekrutieren sie Effektorzellen und aktivieren das Komplementsystem. Einer dieser Antikörper ist Trastuzumab (Herceptin®), ein wachstumshemmender, humanisierter, monoklonaler IgG1 Antikörper. Trastuzumab ist gegen das Tumorantigen HER-2 gerichtet, welches in 30% aller Brusttumore überexprimiert wird. Alle Antikörper, die bis jetzt in Form von passiver Immuntherapie in der Klinik eingesetzt werden, sind ausschließlich der IgG Subklasse zu zuordnen.

Antikörper der IgE Subklasse sind hingegen bekannt für ihre schädliche Funktion in der Typ I Allergie. Es ist jedoch kaum bekannt, dass IgE Antikörper eine anti-Tumor Wirkung haben und diese Eigenschaft für Immuntherapien in der Onkologie ausgenutzt werden könnte. Das Ziel dieser Doktorarbeit war es daher alternative Strategien zur Behandlung von Krebserkrankungen basierend auf IgE Antikörper zu untersuchen, sowie deren Wirksamkeit mit der von IgG zu vergleichen.

Die orale Immunisierungsroute eignet sich sehr gut zur Induktion einer Th2 Immunantwort mit hoch affinen IgE Antikörpern, die gegen das applizierte Antigen gerichtet sind. Daher wird die Etablierung eines IgE-abhängiges Nahrungsmittelallergiemodells in Mäusen beschrieben. Dieses Modell haben wir anschließend für unsere Krebsstudien adaptiert. Mäuse wurden mit unterschiedlichen Konzentrationen von Ovalbumin unter gleichzeitiger Säuresuppression gefüttert. Dies führte zu einer Induktion von antigen-spezifischen IgE in einem Th2 Milieu. Dieses orale Immunisierungsschema wurde auch mit HER-2 Mimotopen, d.h. Peptide, die das Trastuzumab-Epitop imitieren, angewendet. Die durch das Mimotop induzierten IgE Antikörper erkannten spezifisch das Tumorantigen auf HER-2

überexprimierende Brustkrebszellen und führten zur Lyse der Tumorzellen. Ergänzend zu diesem aktiven immuntherapeutischen Ansatz wurde ein Trastuzumab IgE Antikörper für passive Immuntherapie konstruiert. Wir konnten zeigen, dass Trastuzumab IgE die gleiche Spezifität wie Trastuzumab IgG besitzt. In einem Three-Colour Flow Cytometric Assay zeigte sich, dass beide Antikörper Tumorzellen erfolgreich zerstören, dies aber durch unterschiedliche Mechanismen tun: Trastuzumab IgG führte zu „antibody-dependent cell-mediated phagocytosis“ (ADCP), während Trastuzumab IgE „antibody-dependent cell-mediated cytotoxicity“ (ADCC) auslöste.

Ausgehend von den hier präsentierten Daten schließen wir, dass tumor-spezifisches IgE, aktiv induziert oder passiv verabreicht, eine vielversprechende Alternative zu Immuntherapien mit IgG gegen Krebs darstellt.

CHAPTER I

Introduction part I

1. Carcinogenesis

Carcinogenesis in humans is a multistep process reflecting genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivates [1]. Douglas Hanahan and Robert A. Weinberg proposed six capabilities shared by most and perhaps all types of human malignancies: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [2]. Dunn *et al.* suggested a seventh hallmark of cancer, "immunoediting", which makes tumours capable to escape from control mechanisms of the immune system. This process is divided into three phases: elimination (cancer immunosurveillance), equilibrium (cancer persistence) and escape (cancer progression). The immune system differentiates between self versus pathogen and non-self, but also between self and transformed self. However, the immune system does not only protect the host against tumour development, it also forms the immunogenic phenotype of the developing tumour. Therefore the term "immunoediting" describes in an appropriate way the two functions of the immune system during carcinogenesis [3].

In our opinion the fact that the immune system is involved in tumourigenesis gives an optimistic perspective for immunological anti-cancer therapy. By exploiting immune mechanisms we can target and specifically destroy malignant cells while sparing normal cells. In contrast, chemotherapeutic drugs impair mitosis of predominantly fast-dividing cells, but do not differentiate between malignant and normal cells. Therefore they cause numerous side effects, such as derogating the immune system.

Significant gains in cancer therapeutics have been made in the last decade. More potent, less toxic derivates of classic chemotherapeutic agents have been developed and dosing and combination treatments have been optimized. However, the most promising therapeutic advancements are agents targeting proteins encoded by genes that are mutated in malignant cells, such as HER-2, a member of the epidermal growth factor receptor family (EGFR).

2. Epidermal growth factor receptor family (EGFR, ErbB)

Extracellular growth factor ligands bind to receptor tyrosine kinases (RTKs) thereby activating intracellular signalling pathways which result in diverse biological responses including cell proliferation, differentiation, cell motility and survival. The epidermal growth factor receptor family, which is also called ErbB (avian erythroblastosis oncogene B) family, represents one of the human RTKs, which is expressed, with few exceptions (e.g. haematopoietic cells) in cells of mesodermal and ectodermal origins [4].

The ErbB family has four members, EGFR (HER-1 or ErbB1), ErbB2 (HER-2), ErbB3 (HER-3), and ErbB4 (HER-4). It is regulated by various ligands, such as epidermal growth factor (EGF)-like proteins, transforming growth factor (TGF)-α, amphiregulin, betacellulin, epiregulin, heparin binding EGF-like growth factor (HB-EGF) and four known neuregulins (NRGs). The ErbB signalling network evolved through diversification from invertebrates. Its structure and function is highly conserved for more than a billion of years. In the nematode *Caenorhabditis elegans* the simplest form of the EGF receptor system is found, including one receptor and one ligand [5]. In the fruit fly *Drosophila melanogaster* one receptor already interacts with four ligands [6]. Finally, a complex network of 4 receptors and more than 30 ligands is found in mammals [7]. Within this network the four cascades richly interact with each other and establish a layered signalling network. Evolution prefers complex networks

because they are trained to resist perturbation. But they are fragile to uncommon attacks, like recruitment of immune mechanisms by monoclonal antibodies, combination of monoclonal antibodies and chemotherapy or the combination of two different monoclonal antibodies which is currently under investigation in clinical trials [8].

Most data on the ErbB family function in mammals derive from studies with transgenic mice. They showed that this signalling network plays a crucial role in cardiac and neural development [9-15]. The ErbB receptors also play an important role in the adult organism, e.g. in the postnatal differentiation of the mammary gland. HER-1 seems to function in ductal growth, while HER-2 and HER-4 seem to have key roles in lobulo-alveolar differentiation and lactation [16].

Moreover, potent signalling pathways are activated by the ErbB receptors: Ras-MAPK, PI3K/Akt, PLCγ-PKC and STATs [17]. These pathways activate transcriptional programmes in the nucleus, e.g. proto-oncogenes fos, jun and myc, but also zinc-finger-containing transcription factors including Sp1 and Egr1 [4].

In many different cancer cell types, the ErbB pathway becomes hyperactivated by different mechanisms: overproduction of ligands and/or receptors or constitutive activation of receptors.

2.1 Structure of the ErbB receptors and the importance of dimerization

All four ErbB receptors are composed of an extracellular ligand binding domain (~ 620 residues), a single transmembrane segment (~ 23 residues), and an intracellular protein tyrosine kinase domain (~260 residues) that is flanked by juxtamembrane (~40 residues) and C-terminal (~232 residues) regulatory regions. The receptors exist as monomers and their activation depends on ligand binding and other receptors of the same family. Dimerization of

the receptors is necessary for signalling cascade activation. It can occur between two different ErbB receptors (heterodimerization) or between two molecules of the same receptor (homodimerization). 10 distinct homo-and heterodimers can be formed and are activated by different ligands.

The extracellular region of the ErbB receptors is heavily glycosylated and consists of four domains. There are two homologous large domains (L), and two cysteine-rich domains (CR), which occur in the order L1-CR1-L2-CR2 [18], an alternative nomenclature is I-II-III-IV [19]. Ligand binding occurs on domain I and III and leads to receptor dimerization. Almost all receptor-receptor interactions are mediated by domain II. At the center of the dimer interface on domain II is a prominent loop, which has been termed the “dimerization arm”. Deletions or mutations in this region completely prevent ligand-induced activation [20]. EGFR, HER-3 and HER-4 exist in a “closed” conformation in which the dimerization domains cannot interact with each other in the absence of a ligand (inactivated configuration). Via simultaneous binding to both domains, I and III, the ligand alters the orientation of these two domains, thereby changing the conformation of domain II, which finally result in an enhanced ability of the receptor to form dimers (activated configuration) [21]. Because there has not been found any ligand for HER-2 yet, this receptor only exists in an active “open” conformation and is as a consequence permanently available for dimerization [22, 23] **(Figure 1).**

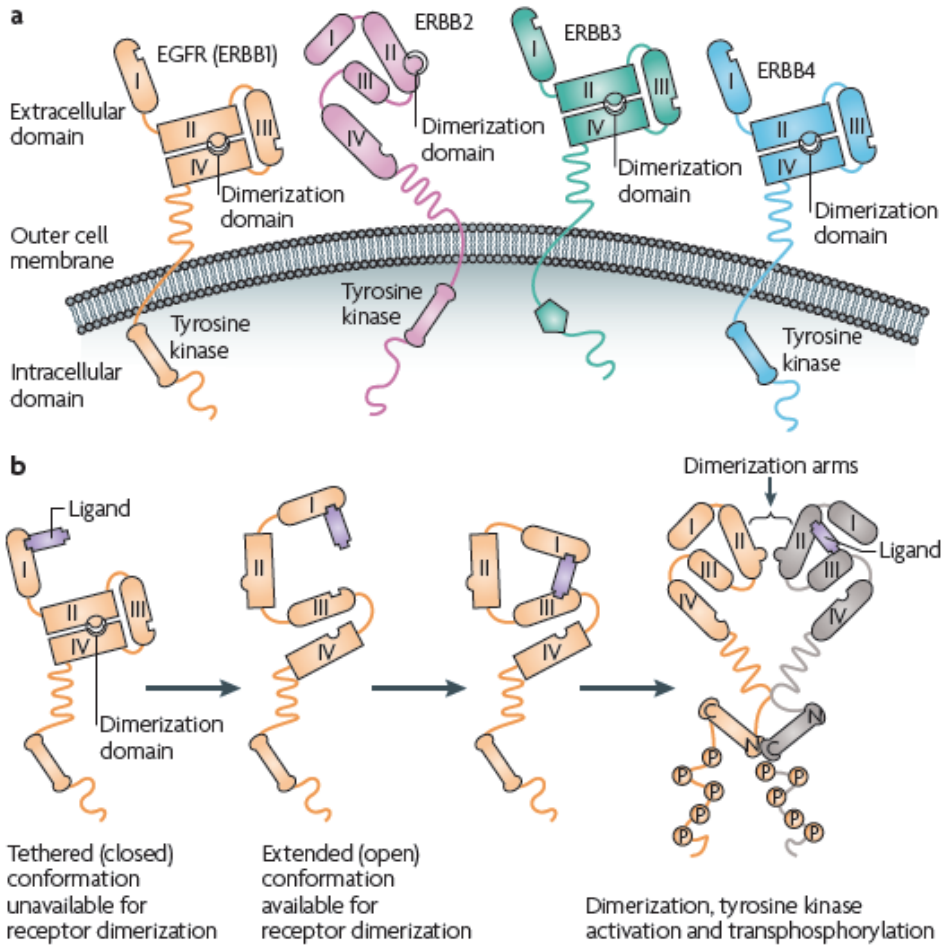

Figure 1. The ErbB receptor family. EGFR (ErbB-1), ErbB-3 and ErbB-4 exist in a tethered conformation, whereas ErbB-2 exist in an extended conformation [23].

2.2 HER-2

The HER-2 receptor is encoded by the HER-2 gene, a proto-oncogene mapped to chromosome 17q21 and represents a 185kD transmembrane glycoprotein [24]. Elevated expression of HER-2 is found in a variety of human cancers, including mammary, bladder, colon, ovarian, gastric, lung and salivary gland tumours. HER-2 positive malignancies correlate with more aggressive tumour phenotypes including tumour size, lymph node involvement, high percentage of S-phase cells, aneuploidy, and lack of steroid hormone

receptors [25]. 20% - 30% of breast cancers overexpress the HER-2 receptor, in ductal carcinoma *in situ* even 60%. In 92% of breast cancer patients HER-2 overexpression is the result of HER-2 gene amplification [26]. However, overexpression can be caused additionally by transcriptional or post-transcriptional dysregulation [27]. HER-2 protein levels are about 10- to 100-fold higher on the cell surface of HER-2-positive tumours compared with adjacent normal breast epithelial cells [28]. Elevated levels of HER-2 expression are associated with aggressive tumour growth and metastatic activity and thus are an indicator of poor prognosis [29]. In human breast cancer, HER-2 gene amplification is correlated with a shorter overall survival and relapse-free survival [29]. Higher levels of the extracellular domain (ECD) of HER-2, which can be cleaved proteolytically from the cell membrane [30] directly correlate with poorer prognosis and a decreased responsiveness to therapies [31].

HER-2 has some unique properties within the ErbB family. As already mentioned it has no high affinity binding ligand and exists in a constitutive open conformation which suggests that this receptor mainly functions as a co-receptor for other ErbB receptors. Interactions between the receptors within the ErbB family are not random, instead, HER-2 is the preferred dimerization partner [32]. Heterodimerization of HER-2 enables the participation of HER-2 in signal transduction although a specific ligand is missing [33]. In general, heterodimers generate more potent signals than homodimers. Particularly, HER-2 containing heterodimers show a high signalling potency, are long-lived and associated with malignant growth. Overexpression of HER-2 promotes the formation of more HER-2 heterodimers, particularly with HER-3. In contrast to HER-2 which has no ligand, HER-3 is devoid of intrinsic kinase activity [34]. Although, neither HER-2 nor HER-3 can support linear signalling alone, the heterodimer of these two is the most transforming and mitogenic receptor complex [35]. HER-2 forming heterodimers with HER-1 lead to a reduced degradation and an enhanced membrane recycling of HER-1. HER-1 homodimers normally undergo degradation in

lysosomes, but when heterodimerized with HER-2 it returns to the cell surface and signalling is prolonged [36].

To inhibit HER-2 mediated signalling different therapeutical approaches are currently under investigation. Monoclonal antibodies, such as trastuzumab and pertuzumab, lead to growth inhibition through different mechanisms dependent on their binding to different epitopes on HER-2. Combinations of antibodies and drugs, e.g. DM1, an anti-microtubuli agent, as well as bi-or trispecific antibodies targeting HER-2 and e.g. CD3 should enhance the efficacy of therapeutic antibodies. Another strategy is the application of small-molecule tyrosine kinase inhibitors (lapatinib), which inhibit the activation of the HER-2 tyrosine kinase domain. Inhibitors of heat shock protein 90 (HSP90), which normally facilitates the folding and conformation of proteins, lead to ubiquitination and degradation of HER-2. Another alternative approach would be to prevent HER-2 proteins from reaching the cell surface, by either blocking their transcription or their trafficking to the cell surface [4, 23]. However, to date the only routinely applied therapy against HER-2 overexpressing breast cancer is based on monoclonal antibodies.

3. Monoclonal antibodies as therapeutic tools in cancer

The vision of antibody-based therapies tracks back to the “magic bullet” concept of Paul Ehrlich. He postulated that selective drugs were needed which have specific affinity for pathogens and act as magic bullets without affecting the host’s cells [37]. The realisation of his vision became more feasible with the generation of the first monoclonal antibodies (mAbs) by Köhler and Milstein in 1975 [38]. However, the murine origin of the mAbs limited their clinical application because they were immunogenic when injected into humans and led to human anti-mouse antibody responses (HAMA). Moreover, due to their murine constant

antibody region, they failed to recruit human effector cells and lacked all functions of the immune system. The upcoming recombinant DNA technology in the early 1980s made it possible to generate mouse-human chimeric antibodies, with a murine variable region and a human constant domain which allowed the interaction with human effector cells and reduced the HAMA responses. Rituximab (MabThera®, Roche in EU; Rituxan®, Biogen Idec/Genentech in USA), a chimeric mAb, directed against CD20 on B-cells, was the first monoclonal antibody approved by the United States Food and Drug Administration (U.S.FDA) in 1997. Chimeric mAbs are 65-90% human proteins, whereas humanized mAbs already consist of 95% human proteins, only a few critical amino acid residues involved in the antigen binding are of rodent origin. The first fully human antibody, panitumumab (Vectibix®, Amgen), is directed against EGFR [39] and approved for the treatment of colorectal cancer. It is derived from transgenic mice whose immunoglobulin loci had been genetically inactivated and replaced with the human immunoglobulin genes [40].

For the development of therapeutic mAbs the selection of the right target antigen is prerequisite. Therefore, four properties of the antigen have to be considered: (1) it must be characteristic for malignant cells and should not be expressed in healthy tissue, (2) it can be measured routinely in clinical patient samples, (3) it correlates with the clinical outcome, and (4) its inhibition results in a clinical response in patients positive to the target antigen [41]. Several tumour-associated antigens (TAA) of hematopoietic cells, including CD20, CD52 and CD33, receptor tyrosine kinases (HER-2, EGFR, VEGF) and other surface proteins like MUC-1, CEA and EpCAM have been chosen as targets for the development of mAbs. A monoclonal antibody directed against one of these antigens harbours two functions: i) via its variable domain it binds specifically to the tumour cell and ii) via the constant part it recruits numerous effector cells and activates complement system to function immunologically on the tumour cells.

A large number of monoclonal antibodies are currently under investigation in early or late stage of clinical trials. To date nine therapeutic monoclonal anti-cancer antibodies are approved by the FDA. They are either murine, chimeric or humanized antibodies, either conjugated to a radionuclide or unconjugated, but they all have in common that they are directed against cell surface proteins of cancer cells and are exclusively of the IgG subclass [42].

3.1 Trastuzumab (Herceptin®)

To generate a therapeutic monoclonal antibody the target antigen has to be chosen carefully. Its accessible location on the cell surface makes HER-2 an attractive target for treatment of HER-2 overexpressing cancers. The sequence of HER-2 was published in 1985 by Coussens *et al* [24]. When a number of different anti-HER-2 antibodies were tested, an interesting result was achieved: some antibodies almost completely inhibited tumour cell growth, while others only partially intervened. Interestingly, some antibodies even led to an enhancement of tumour growth [43]. The murine antibody 4D5 was finally selected for humanization because it had the most promising anti-tumour effects [44, 45]. As a result a humanized IgG1κ monoclonal antibody which was termed trastuzumab (Herceptin®) was developed by Genentech (South San Francisco, CA) [46].

Clinical trials started in 1992 and finally resulted in FDA approval in 1998 for women with metastatic breast cancer overexpressing HER-2. In 1999, the efficacy and safety of trastuzumab was studied in 222 women with HER-2 overexpressing metastatic breast cancer that has progressed after chemotherapy. The patients received a starting dose of 4mg/kg intravenously, which was followed by a weekly maintenance dose of 2mg/kg. The response rate was 15%, the most common side effects were infusion-associated fever and/or chills and

the most clinically relevant adverse event was cardiac dysfunction in 4,7% of patients [47]. In a subsequent study, patients treated with chemotherapy plus trastuzumab showed a 50% overall response rate compared to 32% treated only with chemotherapy, a longer time to disease progression, a longer duration of response, a lower rate of death at 1 year, and a 20% reduction in the risk of death [48]. This therapeutic combination regime was also approved by the FDA. In the Herceptin Adjuvant (HERA) trial study the efficacy of trastuzumab in early-stage breast cancer was analysed. Trastuzumab was given every 3 weeks for 1 or 2 years after adjuvant chemotherapy resulting in an absolute benefit in terms of disease-free survival at two years of 8.4% compared with the observation group [49].

Trastuzumab binds HER-2 on the C-terminal portion of domain IV at the site of the binding pocket for the dimerization domain II. Three loops of HER-2 are in contact with trastuzumab: loop 1 (residues 557-561) and loop 3 (residues 593-603) are primarily electrostatic, whereas loop 2 (residues 570-573) is hydrophobic [50]. Although trastuzumab belongs to the most well studied mAbs, is known to suppress HER-2 signalling, and is already used for passive immunotherapy, the exact mechanisms of its action is still not completely understood. The following mechanisms of action are currently under discussion [23, 51-53]:

(1) HER-2 dimerization is prevented, although it is known that the mAb pertuzumab via binding to the dimerization domain II of HER-2 works more efficiently than trastuzumab.

(2) HER-2 is endocytotically removed from the cell surface which results in degradation of the oncoprotein. As a consequence less HER-2 is present on the cell and less HER-2 heterodimers can be built, thereby reducing signal transduction.

(3) Overexpression of HER-2 on the cell surface leads to proteolytic cleavage of the receptor which results in the release of the extracellular domain and in the production of a truncated membrane-bound fragment (p95), which results in growth signals [54]. Molina *et al.*

demonstrated that trastuzumab can block the shedding of the extracellular domain of HER-2 [55].

(4) Immunological mechanisms: antibody-dependent cellular cytotoxicity (ADCC) reactions are known to be the predominant action of trastuzumab. To perform ADCC reactions the mAb must bind via its Fc part to Fcγ receptors on effector cells (NK cells and monocytes). In genetic mouse models the involvement of the immune system in mAbs action was confirmed: the anti-tumour activity of 4D5 and trastuzumab was almost abolished in FcRγ-/- mice (lacking the stimulatory FcγRIII), while their activity was enhanced in mice lacking the inhibitory receptor FcγRIIb [56]. Whereas rituximab is known to mediate complement-dependent cytotoxicity (CDC) reactions, this function of trastuzumab is controversially discussed.

(5) Induction of cell cycle arrest in G0/G1 phase. Sliwkowski *et al.* showed that HER-2 overexpressed cells treated with the murine 4D5 or its humanized variant result in an increase in the percentage of cells in G0/G1 phase [57].

(6) Reduction of angiogenesis by downregulation of vascular endothelial growth factor (VEGF) [58, 59].

4. Allergy and cancer – is allergy a risk or a protective factor in carcinogenesis?

Today, up to 25% of the western population is allergic, which raises the question if allergic predisposition might have an advantage in evolution. IgE as a widespread, ancient antibody isotype in mammals is best-known for its unwanted reactions in allergy. However, its complete physiological role is not clarified in detail yet, suggesting a natural function of IgE in the protection against tumours.

Allergy and cancer are two completely different diseases increasing worldwide. Allergy involves IgE-mediated hypersensitivity towards harmless environmental antigens leading to rhinoconjunctivitis, hay fever and asthma. The symptoms can range from mild and local (sneezing, coughing, itching) to life-threatening systemic reactions like anaphylactic shock. In contrast, cancer is the consequence of uncontrolled cell growth leading to organ dysfunction and death. Despite these differences the biological relationship between these diseases has been the centre of many studies in the last decades.

Controversial hypotheses exist trying to explain the relationship between allergy and cancer [60]:

(1) Allergy has a protective effect against cancer by enhancing immune surveillance, thus decreasing the chance of mutated cell proliferation (immunosurveillance hypothesis).

(2) Allergy symptoms have been evolved by natural selection to expel toxins, pathogens and foreign particles before they can initiate carcinogenesis (prophylaxis hypothesis).

(3) Allergy is associated with an increased risk of cancer by inducing chronic inflammation and immune stimulation, which was shown in asthmatic patients with a higher risk for lung cancer (antigenic stimulation hypothesis).

For years epidemiological studies try to find a correlation between the incidence of allergies and diverse malignancies. Controversial data vary from studies reporting a protective effect, to studies reporting allergy as a risk factor for cancer and others which found no correlation. The controversial findings may result from different study designs, restricted number of study participants, the failure to control important cancer risk factors, and the way how atopy is defined and measured which is often only based on interviews and questionnaires. The association between allergy and cancer is very complex and depends on the specific allergic and tumour disease. Most epidemiological studies are retrospective case-control studies, while

only the minor part are cohort studies where atopic patients are monitored over a certain time period. Wang *et al.* has reviewed epidemiological studies on this topic since 1985 and found mixed data, but an overall indication that allergy is associated with a reduced risk for cancer [61]. The biggest prospective cohort study on this topic summarized data from 1.1 million US men and women over a time period of 18 years. They found a significantly lowered risk of 10 percent for overall cancer mortality and 20 percent for colorectal cancer mortality among persons with a history of both asthma and hay fever [62]. Despite the methodological problems in the epidemiological studies and the controversial results, there is experimental evidence that allergy might have a protective function in carcinogenesis.

4.1 IgE and its effector cells

IgE is present in serum of healthy individuals in extremely low concentrations, but is tissue bound in high amounts, especially in epithelia and mucosa. The function of IgE is based on the binding to its effector cells via two receptors: the high-affinity FcεRI receptor (K_a= ~10^{10} M^{-1}) and the low-affinity FcεRII or CD23 receptor (K_a= ~10^8 M^{-1}). In comparison to the affinity of IgG antibodies for Fcγ receptors (K_a= ~10^6 - 10^8 M^{-1}) the affinity of IgE for its receptors is much higher [63]. In humans, FcεRI is expressed on mast cells, basophils, eosinophils, macrophages and monocytes and antigen presenting cells, e.g. dendritic cells and Langerhans cells, and also on platelets [64]. The serum half-life of IgE (2-3 days) is very short [65], but when bound in tissue the half-life of IgE is much longer (several weeks) than that of IgG [66].

Receptor-bound IgE directed against a tumour-associated antigen could recruit effector cells to the tumour site. Binding of the IgE-effector cell complex to the tumour antigen would lead to cross-linking of FcεRI and mediator release of the effector cell. Using the IgE-mediated

potent immune reaction and directing it specifically towards malignant cells would induce local inflammation at the tumour site and result in tumour regression. It is known that a high number of inflammatory cells can be attracted into tumours, including eosinophils, mast cells, lymphocytes and macrophages. Besides expressing the high-affinity IgE receptor they contain various substances which can either directly act in a cytotoxic or phagocytic way on tumour cells (IgE-mediated ADCC and ADCP) or recruit other cells detrimental for the tumour.

Nevertheless, most anti-cancer studies have focussed on cytotoxic CD8+ T cells and natural killer cells. Thereupon, dendritic cells drive cytotoxic T lymphocytes (CTLs) towards tumour cells which expose tumour-associated antigens by their major histocompatibility complex (MHC) class I molecules. Sometimes tumour cells try to escape recognition by CTLs and downregulate MHC class I expression. TAA can also be presented by MHC class II molecules on CD4+ T cells by bystander antigen-presenting cells. Th2-mediated anti-tumour immunity can be very effective and appears to be dependent on the production of IL-4, IL-5 and the recruitment of eosinophils [67, 68].

However, tumour-associated macrophages, mast cells and eosinophils exhibit pro- and anti-tumour effects.

Tumour-associated macrophages (TAMs)

Macrophages derive from monocytes which originate in the bone marrow. Monocytes have the potential to migrate out of the bone marrow into the blood stream. Circulating monocytes can leave the blood stream and enter tissue, where they differentiate into macrophages. Tumour-associated macrophages are a major component of tumour infiltrates having the potential to inhibit as well as to promote tumour growth. They secrete a number of different cytokines, such as interferons, interleukins and tumour necrosis factor-alpha (TNFα). Macrophages can execute cell killing via macrophage-mediated tumour cytotoxicity (MTC)

and via ADCC. MTC is antibody independent and involves the direct binding of the macrophage to the tumour cell, thereupon, the macrophage secrets factors such as TNFα and serine proteases which lead to the lysis of the target cell [69]. On the other hand, TAMs are able to secrete mitogenic cytokines such as platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), EGF and transforming growth factor-alpha (TGF-α), thereupon stimulating tumour cell growth and angiogenesis [69]. The presence of macrophages in breast cancer has been correlated with increased angiogenesis and a poorer prognosis [70].

Mast cells

Mast cells derive from a progenitor cell in the bone marrow and migrate into the tissues where they mature depending on the environmental conditions. Two different types of mast cells have been identified: connective tissue mast cells containing tryptase and chymase (CTMC mast cells) and mucosal mast cells containing only tryptase (MMC mast cells). Mast cells have the ability to change their secretion profile according to the microenvironment and after stimulation they secrete preformed or *de novo* synthesized mediators. It is well known that mast cells play a central role in allergic reactions, but it became evident that they are involved in neuro-inflammatory, autoimmune disorders and cancer processes [71]. It has been observed that mast cells accumulate around the margin of cutaneous malignancies [72]. They secrete a lot of molecules that are either beneficial or detrimental to a tumour. FGF-2, heparin, IL-8 and VEGF (neovascularization), NGF, PDGF and SCF (tumour growth), histamine (tumour growth and immunosuppression) and proteases (tissue disruption, metastasis) are mediators with pro-tumour properties. On the contrary, cytokines such as IL-4 inhibits cell proliferation, TNF- α induces tumour cell death, heparan sulfate proteoglycans prevent neovascularisation, and secretion of chondroitin sulphate could stop metastasis [73, 74]. Breast cancer patients with mast cells in their axillary lymph nodes have a longer survival time and a better

prognosis [75-77]. On the other hand, the presence of mast cells around melanomas was correlated with increased neovascularisation, tumour aggressiveness and poor prognosis [78].

The group of Luiten *et al.* generated a murine IgE antibody, G250 IgE, binding an antigen presented on renal cell carcinoma. G250 IgE activated mast cells to release TNF-α and histamine in the presence of tumour cells [79]. In a subsequent study they developed a human/murine chimeric version of this IgE antibody. In experiments with a mast cell-like cell line, the rat basophilic leukemia (RBL) cell line, transfected with the human FcεRI alpha chain (RBL-7TZ), the chimeric IgE led to activation of the RBL-7TZ, and, in a further step to activation of freshly isolated human basophils [80].

Eosinophils

Elevated numbers of eosinophils are best known for their association with allergic diseases and parasitic helminth infections. Eosinophils develop and mature in the bone marrow and are mainly found in tissues secreting a huge number of different products, including granule proteins (major basic protein MBP, eosinophil peroxidase EPO, eosinophil cationic protein ECP, eosinophil-derived neurotoxin EDN), cytokines, chemokines and oxygen metabolites [81, 82]. These cytotoxic granule proteins are known to induce tissue damage and dysfunction, a process which could be harnessed to locally destroy tumour cells [81]. On the other hand, they also attract fibroblasts through the release of TGF-β, FGF-2, NGF and VEGF and play a role in tissue repair, fibrosis and angiogenesis [82]. Eosinophils are known to be elevated in peripheral blood and to infiltrate the tissue in many hematologic and solid human tumours [83, 84]. In one of the first studies of its kind, in 1983, Pretlow *et al.* related the presence of tissue eosinophils in colon carcinoma to a prolonged survival [85] which was confirmed by a study in 1999 [86].

A study of Mattes *et al.* supported the positive effect of eosinophils showing that tumour elimination by Th2 cells in a lung metastasis model of B16-F1 CTL-resistant melanoma is dependent on the influx of eosinophils into the tumour site. Further, tumour clearance was connected with the chemokine eotaxin [68]. Another study showed that eosinophil accumulation occurs independently of T cell mediated immune responses and represents an early inflammatory reaction in carcinogenesis [87]. A recent study on this topic reported that eosinophils express a functional gammadelta TCR/CD3 which is involved in eosinophil cytotoxicity against tumour cells [88, 89].

4.2 Tumour-specific IgE for passive anti-cancer immunotherapy

Anti-cancer immunotherapy with monoclonal antibodies is state of the art. The five Ig subclasses in humans differ in regard to their corresponding effector cells, receptor specificities and mechanism of action. IgG1 is the favoured IgG subclass for therapeutic applications because of its high efficacy in mediating ADCC [90]. Although they are successfully applied in clinical oncology, some limitations have to be considered: inadequate tissue distribution, limited access to tumour cells, short half-life and high costs because of repeated administration.

So far, monoclonal antibodies of the IgE subclass have been neglected for clinical anti-cancer therapy because of their well-known unfavourable function in allergy and the risk of anaphylaxis. Nevertheless, IgE antibodies show a series of unique properties, which harnessed against a specific tumour antigen, could lead to tumour destruction [91]:

- the speed of the immune response
- the higher quality and amplitude of the inflammatory reaction
- the higher affinity to receptors and its longer persistence on cells

- the high sensitivity of IgE-armed effector cells to antigen activation
- the location of IgE effector cells in the tissue

If IgE would be used for immunotherapeutic strategies the risk of any severe side reaction, such as anaphylaxis, has to be minimized by the appropriate choice of tumour target, the expression of only a single epitope on the antigen, highly specific antibodies and little shedding of the antigen from the tumour cells. Moreover, several studies have so far described that natural IgE targeting TAAs can be found in malignant lesions. None of these patients has experienced anaphylaxis [92].

One of the first studies on the effects of tumour-specific IgE used a monoclonal murine IgE antibody recognizing an envelope glycoprotein on the mouse mammary tumour virus (MMTV). Passive application led to the protection of syngeneic mice against a lethal dose of H2712 mammary carcinoma secreting the MMTV virus. However, the mechanism behind the anti-tumour effect of IgE and the involved effector cells were not discussed in this study [93]. The biological activity of tumour-specific monoclonal IgE, mIgE 30.6, was investigated in a human tumour xenograft model in SCID mice. Animals were inoculated subcutaneously with colorectal tumour cells COLO 205 and after 5 days they received a single injection of the murine IgE antibody. Tumour growth and tumour size was inhibited specifically in comparison with a chimeric antibody recognizing the same colon cancer associated antigen, but consisting of a human Fc part [94]. The construction of a human monoclonal IgE antibody directed against the folate binding protein expressed on human ovarian carcinoma was the next step forward towards clinical application. Thereby MOv18-IgE was directly compared with its analogue MOv18-IgG. In a SCID mouse xenograft model of ovarian carcinoma the beneficial effect of IgE was greater and of longer duration than that of IgG. No signs of anaphylactic reactions were observed in any of the animals. In a subsequent study the authors found that human monocytes were very active in IgE-mediated ADCC. Furthermore, it was

speculated that the IgE could be even more efficient in human patients than in the SCID mouse model because human PBMC were rapidly cleared from the circulation of mice, while in humans the effector cells would be permanently available [91, 95].

These studies confirm the hypothesis that IgE has the potential to trigger inflammatory and allergic reactions at tumour sites by the recruitment of potent tumouricidic cells.

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Introduction part II

Review

Mimotope vaccination – from allergy to cancer

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Review

Expert Opinion

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Mimotope vaccination – from allergy to cancer

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Background: Mimotopes are peptides mimicking protein, carbohydrates or lipid epitopes and can be generated by phage display technology. When selected by antibodies, they represent exclusively B-cell epitopes and are devoid of antigen/allergen-specific T-cell epitopes. Coupled to carriers or presented in a multiple antigenic peptide form mimotopes achieve immunogenicity and induce epitope-specific antibody responses upon vaccination. *Objective/methods*: In allergy IgG antibodies may block IgE binding to allergens, whereas other IgG antibody specificities enhance this and support the anaphylactic reaction. In cancer, inhibitory antibody specificities prevent growth signals derived from overexpressed oncogenes, whereas growth-promoting specificities enhance signalling and proliferation. Therefore, the mimotope concept is applicable to both fields for epitope-specific vaccination and analysis of conformational B-cell epitopes for the allergen/antigen. *Results/conclusions*: Mimotope technology is a relatively young theme in allergology and oncology. Still, proof of concept studies testing allergen and tumour mimotope vaccines suggest that mimotopes are ready for clinical trials.

Keywords: allergooncology, allergy, cancer, epitope specificity, immunotherapy, mimotope, vaccination

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1. Introduction

In both allergy and cancer biology, antibodies may be beneficial or detrimental, depending on their epitope specificity [1,2]. In allergy, specific immunotherapy (SIT) with allergens aims to induce antibodies which block, but do not enhance the allergic reaction. Similarly, in immunological targeting of antigens overexpressed by malignant cells, growth-inhibitory antibodies are preferred, whereas growth-stimulating specificities should be prevented. Therefore, in both cases it is important to direct immune responses to inhibitory antibody epitopes of the allergen/antigen. Phage display technology enables the selection of mimotopes that structurally mimic such B-cell epitopes. These mimotopes can be used to localize conformational epitopes and, more importantly, for active immunization strategies. The induced antibodies directed against the native allergen/antigen represent a polyclonal humoral immune response with memory function. Thus, mimotope technology opens new avenues for epitope-specific treatment of allergy and cancer.

2. Current status of allergy treatment

Today, an average of 23% of the population in industrialized countries suffers from seasonal allergic rhinitis [3]. Thereby IgE antibodies, elicited against harmless antigens, cause mild to severe symptoms and even life-threatening anaphylactic reactions. Current clinical strategies for the prevention of allergic symptoms involve allergen avoidance by patients, pharmacotherapy or SIT. In 1911 Noon and Freeman reported for the first time, to our knowledge, the efficacy of immunotherapy for the treatment of type I hypersensitivity [4]. For almost a century the principle of therapy has not changed much and is routinely applied against airborne

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allergens and hymenoptera venom [7]. The treatment, accepted by the WHO, comprises repeated administration of increasing doses of allergen extracts to actively induce tolerance towards allergens in patients suffering from rhinoconjunctivitis and asthma [5,6][8]. The efficacy of SIT is clinically well-documented, but the molecular background including humoral and cellular mechanisms, is to date incompletely understood [9].

During treatment an increase of allergen-specific IgG antibodies [10], particularly IgG4 and IgG1 isotypes [11], is observed, sometimes accompanied by a decrease of allergen-specific IgE serum levels. Later it was shown that the increase of allergen-specific IgG antibody levels towards the individual allergens in an extract and the alteration of specific IgE do not necessarily correlate with the outcome of therapy [12]. Furthermore, the exact determination of allergen-specific IgG is even complicated by the presence of anti-IgE autoantibodies [13]. Wachholz *et al.* reported that IgG antibodies induced by grass pollen immunotherapy were able to block the IgE-facilitated antigen uptake by B-cells and subsequently the presentation of allergens to T-cells [14]. This refers to the fact that, beside the humoral anti-allergen response, allergen-specific T-cells also play an important role in allergic inflammation. In contrast to conformational epitopes of B-cells, T-helper cells recognize short linear fragments of allergens which are presented by HLA class II molecules on antigen presenting cells (APCs). In sensitized healthy individuals, regulatory T (T_{reg}) cells are the dominant subset specific for allergens, whereas patients with allergy show a bias towards allergen-specific T helper type 2 (T_H2) cells. The induction of a tolerant state in peripheral T-cells represents an essential step in SIT [15]. On the cellular level the induction of IL-10-producing T_{reg} cells [16], and the shifting from a T_H2 to a T_H1 cytokine profile [17] are known to contribute to a successful immunotherapy.

SIT harbours a certain risk for side effects, such as the possibility of large local inflammatory [18] and anaphylactic reactions [19]. Allergen extracts used for SIT are prepared from natural allergen sources and show high variation in their protein composition and amounts. They consist of allergenic and nonallergenic components [20,21], therefore they cannot be adjusted precisely to the patient's profile. Subsequent induction of new sensitization to irrelevant components has been observed after this treatment. Thereby, IgE antibodies are produced *de novo* against previously unrecognized B-cell epitopes on the allergen [22-24]. To make SIT more safe several attempts have concentrated on the concept of component-resolved diagnosis and therapy, which is based on recombinant allergens and hypoallergenic allergen derivatives. The advantage of recombinant allergens is the production of molecules with defined quality devoid of irrelevant components, allowing treatment according to the patient's sensitization profile [25]. Nevertheless, recombinant allergens still may present novel epitopes that possibly elicit new IgE antibody specificities. To bypass this unwanted reaction other studies focus on the concept of epitope-specific immunotherapy.

2.1 The importance of epitope specificity in allergy

The blocking capability of IgG is due to an antigen trapping or due to a blockade of the IgE-facilitated allergen presentation by B-cells to T-cells [14,26]. Furthermore, co-crosslinking of FcεRI and FcγRIIb receptors by allergen-IgG complexes can negatively regulate allergy effector cells [27]. To be able to compete with IgE being a high affinity antibody, one would expect that high affinity of the IgG to the allergen represents a desirable feature of a potential blocking antibody. In surface plasmon resonance studies, however, none of the tested purified human or murine monoclonal IgG antibodies were able to reach the affinity of IgE antibodies to allergens [28]. Moreover, an affinity maturated anti-allergen IgG antibody recognizing the wrong epitope will, even though present in molar excess, not successfully interfere with the binding of IgE to the allergen [29]. It was demonstrated that among different monoclonal antibodies specific for birch pollen major allergen Bet v 1 some antibodies were able to block IgE binding to Bet v 1, whereas others were able to even promote IgE recognition of Bet v 1 [30] *in vitro* [1] and *in vivo* [31]. The mechanism might be linked to the promotion of homodimerization and thus the IgE crosslinking capability of allergens [32] (**Figure 1**).

Consequently, SIT with whole allergen extracts or recombinant proteins, respectively, will produce both types of anti-allergen IgG, anaphylactogenic and blocking antibodies.

Therefore, it is crucial to systematically analyse the structural details of IgE epitopes on allergens. In principle, epitopes can be either of the linear (continuous) type, or of a conformational nature. Conformational, discontinuous epitopes are formed when amino acid residues that are not neighbouring within the sequence become spatially juxtaposed in the folded protein. For several allergens it could be demonstrated that effective IgE binding depends on their three-dimensional structural integrity [33]. In contrast, linear epitopes are formed by adjacent amino acid residues and may be inaccessible in the native conformation. A number of studies have tried to delineate B-cell epitopes from the primary amino acid sequence of proteins. This strategy is limited to the identification of solely linear epitopes, whereas B-cell epitopes cannot be predicted from these analyses because of their conformational nature [34]. Epitope-mimics, so-called mimotopes, represent a close approximation of the original 3D-epitope, even though their amino acid composition rarely shows similarities. This is due to the fact that mimotopes mimic an epitope by their biochemical and electrostatic properties, and not necessarily by sequence homology [35]. With the phage display technology it is possible to generate such structural mimics of B-cell epitopes.

3. Current status of cancer treatment

Whereas active immunotherapy is the preferred option in allergy treatment today, in cancer therapy passive immunotherapy is state of the art. Advances in recombinant DNA technology

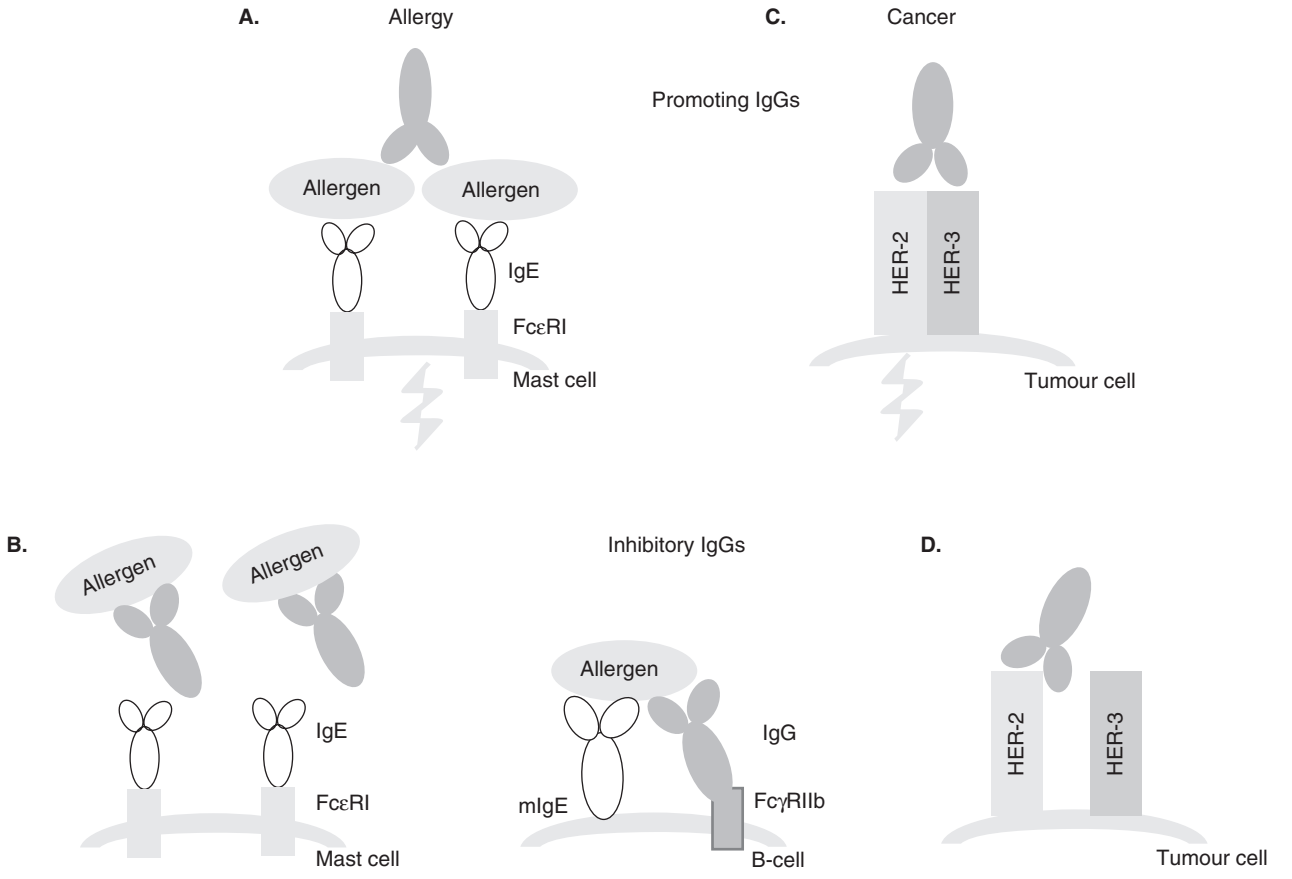

Figure 1. The effects of IgG in allergy and cancer. Depending on their epitope specificity IgG antibodies can either inhibit or promote allergic reactions or tumour growth. **A.** In allergy, specific IgGs enhance the IgE binding to allergens and the anaphylactic reaction, which could be due to supporting the crosslinking capability of allergens. **B.** On the one hand, inhibitory IgG antibodies which recognize the IgE epitope on the allergen directly block the binding of IgE to the allergen, thus, preventing mediator release. On the other hand, IgGs can act via the FcγRIIb receptor: On B-cells this results in an inhibition of IgE-facilitated antigen presentation. The inhibitory effect on FcεRI positive effector cells is not illustrated. **C.** In cancer, heterodimerization of HER-2, HER-3 and other family members leads to signalling and proliferation. Therefore, antibodies promoting heterodimerization are detrimental. **D.** In contrast, IgG antibodies hindering heterodimerization prevent signal transduction and cell growth.

enabled the development of chimeric and humanized monoclonal antibodies that are far less antigenic than the original mouse monoclonal antibodies and exhibit longer half-lives within the serum [36]. With this invention a new area of anti-cancer therapy based on monoclonal antibodies additional to the classical treatments of cancer including surgery, radiation and chemotherapy began. Targets for monoclonal antibodies are growth factor receptors and surface protein and non-protein antigens overexpressed on malignant cells. Furthermore, antibodies have been armed with cytokines, chemotherapeutic agents, toxins, and radionuclides to augment their tumouricidic efficacy [37]. In 1997, rituximab (MabThera®, Roche in EU; Rituxan®, Biogen Idec/Genentech in USA) directed against CD20 on B-cells for the treatment of non-Hodgkin's lymphoma, became the first approved monoclonal antibody; since then nine monoclonal antibodies have received approval from the United States (FDA) to date for anti-cancer therapy, all of them of the IgG class [38,39].

One of the clinically most used monoclonal antibodies, trastuzumab (Herceptin®, Roche), is directed against the extracellular domain of human epidermal growth factor receptor 2 (HER-2) [40,41], a member of the EGFR family. HER-2 constitutively exists in an activated conformation [42], lacks ligand-binding activity and its signalling depends on heterodimerization with other members of the EGFR family, preferentially with HER-3 [43,44]. Trastuzumab was originally selected from a series of monoclonal antibodies, among which were detrimental, growth-promoting species. Trastuzumab acts via mediating antibody-dependent cellular cytotoxicity (ADCC), blocking the shedding of the extracellular domain of HER-2, reducing HER-2 signalling by physically inhibiting homo- or heterodimerization, initiation of G1 arrest and inhibition of angiogenesis [45,46].

Generally, high costs, repeated administration to achieve sufficient antibody titres and anti-tumour activity, as well as inadequate tissue distribution in the patients are the limiting factors in passive immunotherapy. An alternative, more patient-friendly approach is active immunotherapy with mimotopes, producing a polyclonal immune response consisting of the desired antibody specificity and additionally stimulating immunologic memory.

3.1 The importance of epitope specificity in cancer

From the above it is clear that epitope specificity plays a crucial role in the development of immunotherapy with monoclonal antibodies. To focus on trastuzumab, one has to mention that originally a number of anti-HER-2 antibodies had been isolated [47]. Among them, 4D5 (later trastuzumab) was selected due to its growth-inhibitory functions [48], in contrast to others, which even stimulated cancer cell growth [49]. The difference in their biological activity is strictly dependent on the respective targeted epitope on HER-2 and has to do with their ability to hinder or promote heterodimerization of HER-2, the latter inducing signal transduction and cell proliferation [2] (**Figure 1**). For this reason the characterization of tumour antigen epitopes is a basic step in developing epitope-specific immunotherapy. Yip *et al.* identified large and conformational B-cell epitopes on the tumour antigen HER-2 [2]. In 2003 Cho *et al.* successfully defined the binding site of trastuzumab as an epitope composed of three loops on the HER-2 molecule [50]. The work by Riemer *et al.* [51] showed that it is feasible to imitate such complex epitopes using peptide mimics generated by phage display technology: five characterized HER-2 mimotopes indeed matched to loop three, as defined by Cho *et al*.

4. Phage display technology

Phage display technology, first introduced by G. Smith in 1985 [52], is an advanced tool to define peptide structures that mimic natural epitopes including both conformational and linear epitopes. These peptides are called mimotopes [53]. The strength of the phage technology lies in the display of up to 10^9 different peptides in a library form enabling the selection of mimotopes in a repetitive screening procedure. A large number of protein–antibody, virus–antibody and ligand–receptor interfaces have already been mapped by the use of phage display technology [54].

For the creation of a peptide phage display library random oligonucleotides are inserted into the genome of the filamentous bacteriophage fd using either the minor coat protein pIII (display of 3 – 5 copies/phage) [52] or the major coat protein pVIII (display of up to 2700 copies/phage) [55] as display system on the surface of the phages. The peptides can be presented in either linear or circular form. To construct circular peptides the sequence has to be flanked by two cysteine residues. By building a disulfide bond a constrained cycle is formed and presented on the phage surface [56]. Beside the different structural presentations, the length of the peptides can vary from 6 to 38 amino acids [57]. Thus, within this phage system only the presentation of relatively short peptide sequences is possible, whereas larger gene insertions of proteins or antibody fragments appear to prevent pIII and pVIII functions necessary for phage reproduction [57]. To overcome this problem phagemid systems have been developed [58]. The phagemid itself carries only the phage gene gIII or gVIII, containing the foreign sequence, and needs a helper phage with all the necessary genes for phage production including also a copy of the wild-type gene used for display. Therefore, both recombinant and wild type proteins will be produced and incorporated into the phage particle. For pIII one of five copies, and for pVIII from 1% to 30% of the 2700 copies will display the foreign protein [59]. For the production of combinatorial antibody libraries primarily phagemid systems are used resulting in the display of 'giant mimotopes', most often in association with pIII. Antibody display can be performed: i) by antibody fragment (Fab) systems, including two light chain domains (variable and constant) and the variable and first constant domain of the heavy chain; ii) by single-chain variable fragment (scFv) systems where only the variable domains of each chain are presented; or iii) by single-chain Fab (scFab) [59].

4.1 Biopanning-selection and characterization of the mimotope

In 'biopanning' [60] allergen- or antigen-specific antibodies of interest, monoclonal or polyclonal, are adsorbed on microtiter plates and incubated with a phage display library containing the complete repertoire of the respective library. Phages displaying peptide or protein domains which bind to the antibodies are caught whereas unbound phages are removed by washing steps. Bound phages are then eluted from the complexes by acidic solutions (such as HCl or glycine buffer) or by competition with the original antigen, if available. Amplified phage particles of the preceding round are used as starting material for the next panning round. Thereby, specifically interacting ligands can be amplified with great efficacy. Eluents from the biopanning rounds are tested by ELISA or other immunological methods. An increase in the titre of phage particles specifically binding to the selection antibody during the panning rounds is a first indicator of successful selection. Subsequently, the colony screening method is used to identify specifically interacting phage clones for further analysis. Generally, a strong signal in these tests predicts good mimicry of the original antigen, but this has to be additionally proven by competition assays with the original antigen or allergen [61]. After sequencing the most promising clones, computational matching studies can be performed using a software program rendering visualization of the location and the structural features of the epitope of interest [51,62]. Subsequent immunization studies with the mimotopes must prove molecular mimicry and

should lead to antibodies recognizing the original allergen/antigen. **Figure 2** illustrates the principle of mimotope selection, starting from the choice of the antibody used for biopanning to the desired allergen/antigen-specific immune response in mice.

5. Utilizing mimotopes to localize epitopes by computer modeling

Several groups have invented algorithms and programs that assist epitope mapping. They work with mimotope and antigen sequence information [63], or with mimotope sequence and antigen structure [64-66], or integrating the different approaches. A recent publication presents the Pep-3D-Search approach for predicting the epitope area through mimotopes or a motif sequence derived from a set of mimotopes [67]. Among all, MIMOX was the first free web tool for structural mapping of an antibody epitope by mimotope information [68].

During our own early work with mimotopes these programs were not available and we were forced to develop different methods to approach the question of 3D-epitope mapping: i) In an attempt to characterize a structural IgE epitope on birch pollen allergen Bet v 1, a computer-aided, 3-dimensional coarse-grained epitope search was developed [69]; ii) More recently, when we characterized the IgE epitopes of house dust mite allergens Der p 1 and Der p 2 [62] epitope matching was performed using BALL (Biochemical Algorithms Library) [70]. Two criteria were used to assess the quality of the potential epitope: solvent exposure and amino acid similarity assessed by a modified Gonnet matrix [71]; and iii) Even more challenging was the alignment of mimotopes to HER-2 due to the 10 times larger molecular size than with allergen molecules resulting in the need for higher calculation power. Based on the antigen molecule's spatial structure from the RCSB Protein Data Bank [72] the algorithm took into account the relative flexibility of short peptides when scanning a given protein surface for matching sites [51]. Further, for investigations of mimotope–antibody interactions we took advantage of AutoDock 3.0.5 [73]. With this program it is possible to dock the oligopeptides to the Fab fragment of interest [35]. Taken together, the computational approaches available today allow allocation of B-cell epitopes with a high predictive accuracy.

6. Making the mimotope immunogenic

After mimotope characterization the obtained information must be applied to design an immunogen for successful induction of an epitope-specific immune response. Peptides are too small to elicit an immune response and are, therefore, immunologically ignored [74] or may induce tolerance [75]. Phage particles themselves are highly immunogenic because they carry sufficient T-helper cell epitopes on their surface [76]. On the other hand, phage-displayed mimotopes are not suitable for clinical studies in humans because the phage particles carry foreign DNA and antibiotic resistances. Therefore, alternative carrier systems have to be chosen which are necessary for achieving sufficient immunogenicity for vaccination.

Keyhole limpet hemocyanin (KLH) is a large protein that acts as a carrier, causes a strong immune response and provides additional T-helper cell epitopes, which is important as T-helper cells B-cells stimulate to increase their production level and quality of antibodies [77]. In parallel, also cytotoxic T-lymphocytes (CTLs) may be activated through T-helper-cell-derived IL-2. Tetanus toxoid (TT) represents a carrier protein similar to KLH with T-helper activity and has also been used as a peptide carrier previously [78,79]. In order to prevent crosslinking of preexisting IgE in allergy, the choice of the appropriate carrier system of an allergen mimotope has to be taken into consideration when constructing a vaccine. In an allergology study albumin binding protein (ABP) was used to construct a monovalent fusion protein with a mimotope of the birch pollen allergen Bet v 1 [80].

Another option to present a mimotope in a way suitable for immunization is the multiple antigenic peptide system (MAP), where linear peptides are synthesized in a straightforward manner on a tyrosine backbone allowing a dense display of the peptides [81]. Recently, we synthesized a linear mimotope for the carcinoembryonic antigen (CEA) in MAP form and termed it "multiple antigenic mimotope" (MAM). This CEA–MAM was used for successful anti-cancer vaccination in a murine tumour transplant model [82]. Generally, for the vaccination with mimotopes one has to consider that the orientation and the structure of the mimotope in the vaccine should be the same as it was when displayed on the phage during biopanning. **Table 1** summarizes the advantages and disadvantages of mimotope vaccination.

7. Epitope-specific vaccination with mimotopes

7.1 Mimotopes in allergy

Phage display technology was first applied in allergology to isolate a mimotope of the plant panallergen profilin by performing a biopanning experiment with purified IgG/IgE antibodies from the serum of profilin allergic patients. Constrained and linear random nonapeptide libraries were screened with the anti-profilin antibodies. The selected mimotope represented a cross-reacting epitope mimic for IgE antibodies directed against mugwort, birch pollen and celery tuber profilin and it was concluded that therapies with such peptides might block the antigen binding sites of IgE without cross-linking and subsequent mediator release [83]. Consecutively, a similar panning experiment was performed with two murine monoclonal IgG antibodies, BIP1 and BIP4, directed against the major birch pollen allergen Bet v 1 [84], and with purified IgE from birch-pollen-allergic patients. As expected, alignments with the Bet v 1 amino acid sequence showed no linear homologies, but the epitopes could be localized by a computer-aided mathematical approach based on three-dimensional

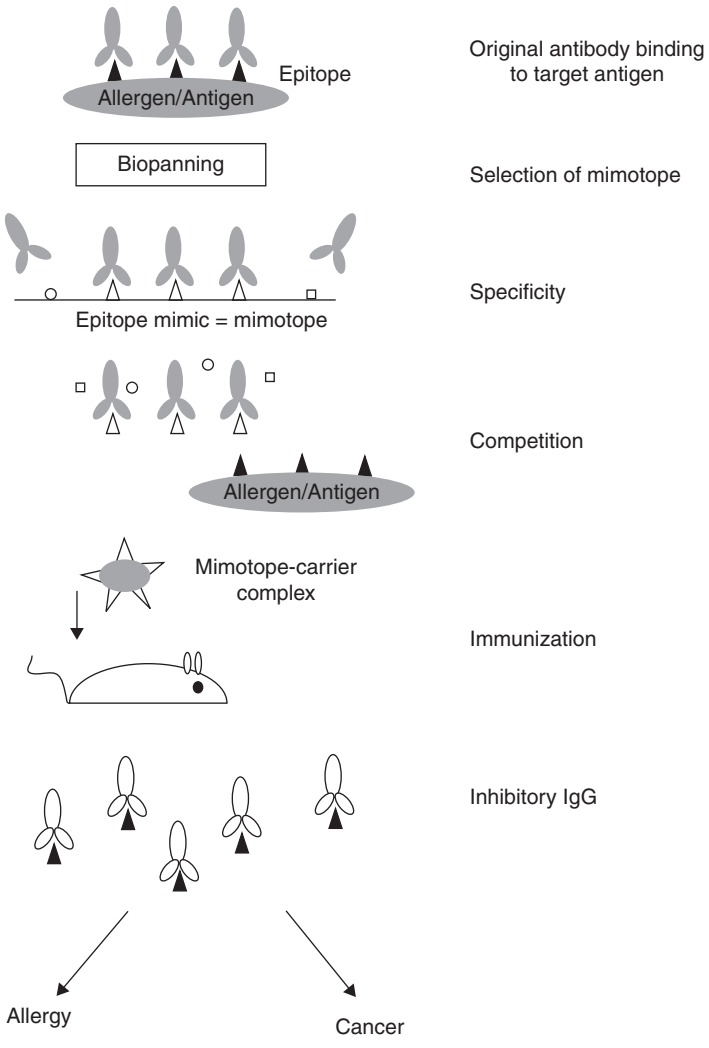

Figure 2. The principle of mimotopes. The first step in generating mimotopes is the choice of an antibody recognizing the allergen/antigen of interest. In allergy, biopanning can be carried out with allergen-specific IgE whereas in cancer, an inhibitory monoclonal antibody against the tumour antigen of interest is chosen. During biopanning mimotopes are selected and further investigated for specificity to the original antibody. Afterwards, mimotopes are tested for their ability to compete with the native allergen/antigen for binding to the original antibody. The selected mimotope is coupled to a carrier and used for immunization studies. After successful immunization, mice produce antibodies against the same epitope of the original allergen/antigen which is recognized by the antibody used for biopanning.

structure and chemical character of the amino acids [69]. Immunization with IgE mimotopes coupled to ABP in a monovalent fashion did not elicit type I skin reactions and, furthermore, induced allergen-specific IgGs that were able to inhibit IgE binding to Bet v 1 [80]. Again indicating the importance of epitope specificity, immunization with the BIP1 mimotope yielded anaphylactogenic antibodies [31].

Besides Bet v 1, which is recognized by 95% of birch pollen allergic patients, Phl p 5, the major grass pollen allergen, represents another important target for immunotherapy in allergy. Using Phl p 5a-specific IgE antibodies purified from allergic patients and screening a peptide as well as a combinatorial Fab library, conformational IgE epitopes could be identified with the help of the selected mimotopes. Further, Fabs and peptide mimotopes induced anti-Phl p 5a-specific immune responses in mice [85,86], even when injected in a DNA minigene format [87].

Furthermore, B-cell epitopes were successfully defined for parvalbumin, the major fish allergen [88] and for the two major house dust mite allergens, Der p 1 and Der p 2 [62]. The latter confirmed a previous study defining a Der p 1 epitope by a biopanning approach with a non-constrained 15mer- and a constrained 9mer-peptide library [89]. The selected Der p 1 and Der p 2 mimotopes determined epitopes within the conserved

Table 1. Advantages and disadvantages of mimotope vaccination in allergy and cancer.

Advantages of mimotopes
Easily obtained by the phage display technology
High stability in storage
Cheaper than the production of monoclonal antibodies
Free of contamination with toxic substances (e.g., from expression systems)
Coupling to immunogenic carrier molecules enhancing their antigenicity
Induction of an epitope-specific humoral immune response
Do not contain allergen/antigen T-cell epitopes
Induction of a long-lasting immune response/ no turn off
Disadvantages of mimotopes
Induction of a long-lasting immune response/ no turn off
Lower affinity than original antibody

regions of the allergens. As crossreactive allergens not only share similar amino acid sequences but also structural homology, the generation of such mimotopes could be useful for the development of SIT targeting epitopes within a clinically cross-reactive allergen group [62]. A recent study identified two relevant conformational IgE-binding regions of Pru p 3, the major peach allergen, using a 12mer-random peptide phage display library [90].

The role of T-cell help for the formation of allergen-specific antibodies after immunization with phage-displayed mimotopes was investigated in proliferation studies with murine splenocytes. As expected, it was shown that the 11mer-mimotope did not crossreact with the allergen at the T-cellular level. It is tempting to speculate that this might be an ultimate advantage, as allergen-specific T-lymphocytes, which nourish chronic allergic inflammation [91] cannot be activated by the applied mimotopes. However, phage-displayed mimotopes were able to drive spleen cells to proliferation indicating that the carrier contributes to the immune response in a mimotope vaccine by recruiting bystander T-cells that help B-cells to generate an appropriate humoral immune response [92].

7.2 Mimotopes in cancer

Much more than in allergy, mimotope work has accelerated in cancer research since the first application of this technology in 2000: one group screened a phage display library with the mouse monoclonal antibody BCD-F9, which recognizes an unknown antigen found on the surface of many tumour cells. After four rounds of biopanning they selected one linear decapeptide, which was able to inhibit the binding of BCD-F9 to HT-1080 fibrosarcoma cells. Moreover, the polyclonal antibodies generated by immunization of rabbits bound specifically to HT-1080. Importantly, in a tumour metastasis model with CD-1 nude mice, passively applied anti-mimotope sera led to a prolonged life span [93].

7.2.1 Tumour-associated carbohydrate antigens (TACAs)

Carbohydrates are primarily T-cell independent antigens that elicit low antibody responses and poor memory. The immunogenicity of these antigens has to be enhanced either by coupling to an appropriate carrier or by turning the carbohydrate into a peptide structure mimicking the original antigen. Mimotopes for different carbohydrate antigens such as GD3/GD2, Lewis Y and sialyl-Lewis X have been developed [94]. GD2, a disialoganglioside, is a relevant tumour antigen expressed on various tumours including neuroblastomas. Using the chimeric anti-GD2 antibody 14.18, which is applied for diagnostic purposes and had already entered clinical trials [95], a biopanning screening was performed. The selected circular mimotopes were analysed in a computer modelling program and fit well into the GD2 binding groove of an anti-GD2 Fab antibody [35]. In the subsequent study these mimotopes were coupled to KLH and administrated as active immunotherapy in mice, inducing a GD2-specific humoral immune response [96]. These and other GD2 mimotopes have been successfully used for immunization in DNA form [97,98]. The DNA-vaccine-induced antibodies recognized GD2-positive tumour cells, mediated complement-dependent cytotoxicity (CDC) and exhibited a reduction of GD2-positive melanoma growth in SCID mice after passive administration [98].

7.2.2 HMW-MAA and Mel-CAM in melanoma

In melanoma, the human high-molecular-weight melanoma-associated antigen (HMW-MAA) [99-101] and the melanoma cell-adhesion molecule (Mel-CAM) [102] have been used for mimotope vaccination studies.

In parallel studies the anti-HMW-MAA monoclonal antibody 225.28S which is known to suppress the metastatic potential of melanoma cells [103,104] was used for mimotope selection in biopanning experiments. We generated a mimotope-fusion protein with ABP and immunized BALB/c mice [100], whereas the group of Wagner *et al.* [99] performed immunization studies in rabbits administrating mimotopes coupled to TT. Independently, both groups showed a specific anti-HMW-MAA anti-body response blotted and on the surface of intact melanoma cells. Even more important, the elicited antibodies inhibited the *in vitro* growth of melanoma cells and exhibited ADCC potential. Both studies demonstrated the principal potential of a mimotope vaccine in melanoma. In 2008, Wagner *et al.* published a study where they purified anti-HMW-MAA antibodies in rabbits after mimotope immunization and tested them *in vivo* in a SCID mouse melanoma xenograft model. A prophylactic and a therapeutic approach revealed that tumour growth was inhibited significantly [105].

Monoclonal antibody MAd18-5D7 against the melanoma antigen Mel-CAM was used for generation of mimotopes using a 28mer-peptide library. The authors showed that the 28mer- mimotope peptides alone were sufficiently antigenic to elicit a specific IgG response against Mel-CAM, but at lower titres than obtained with peptides linked to TT as a

carrier. They further observed the production of T_H1-type cytokines from mimotope-stimulated proliferating spleen cells [102]. This may be due to mimotopes acting themselves as T-cell epitopes after antigen processing and presentation.

7.2.3 *Growth factor receptors*

Numerous studies in cancer have concentrated on the epidermal growth factor receptor family including four members, HER-1 (EGFR, ErbB1), HER-2 (ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4), which are important for regulation of cell proliferation and differentiation [106]. Early studies using phage display methodology with three different tumour inhibitory anti-HER-2 antibodies indicated that due to the absence of sequence homology the epitope was most probably of conformational nature [107]. Our group has focused on the production of mimotopes for EGFR and HER-2 and, performed biopannings with the monoclonal antibodies cetuximab (Erbitux®, MerckKGaA) and trastuzumab [108,109]. Both antibodies are currently applied for passive immunotherapy: cetuximab is approved for therapy in EGFR-overexpressing advanced colorectal cancer and trastuzumab for the treatment of patients with metastatic breast cancer overexpressing HER-2 [38]. In both cases, the mimotope-induced antibodies mediated ADCC and CDC reactions, receptor internalization from the cell surface and a dose-dependent inhibition of tumour cell proliferation in highly specific manner and at a similar intensity to the original antibodies [108,109].

Besides the biopanning approach various groups have defined peptide mimics of tumour B-cell epitopes via computer-aided analysis. Dakappagari *et al.* designed *in silico* B-cell epitope peptides of HER-2, facilitated their folding mimicking the native protein structure and synthesized them collinearly with a promiscuous T-helper cell epitope derived from the measles virus fusion protein. Active immunization with one of these peptide constructs prevented the development of tumours in mice transgenic for the rat *neu* gene [110]. Alternatively, they combined two HER-2 B-cell epitope constructs, applied this multi-epitope vaccine in combination with IL-12 as a T_H1 adjuvant and observed a significant reduction in the number of HER-2 positive lung metastases [111].

Another group also generated HER-2 peptides representing B-cell epitopes of HER-2, which specifically induced anti-HER-2 antibodies able to mediate ADCC reactions [112]. This multi-peptide vaccine coupled to TT significantly delayed tumour formation and progression *in vivo* in FVB/N mice carrying the activated rat c-*neu* oncogene, under the control of a mouse mammary tumour virus promoter (MMTV-c-*neu* mice), which develop spontaneous breast tumours. Furthermore, the co-administration of IL-12 led to elevated IFN-γ levels and an increase in IgG2a antibody titre supporting the anti-tumour activity [113].

7.2.4 *CD20*

Another interesting tumour target is CD20 which is expressed on all mature B-cells, but not on their progenitor cells. Therefore, monoclonal antibodies to this antigen will destroy both benign and malign mature B-cells, but allow the progenitor cells to re-establish normal hematopoietic cell lines [114]. Li *et al.* have generated mimotopes of CD20 using rituximab in a biopanning approach. They screened a 12mer peptide library and coupled the best mimotope with respect to mimicry potential, to KLH and TT for immunization experiments in BALB/c mice. With immunofluorescence staining and flow cytometry assays they showed that the immunized mice produced antibodies recognizing the native CD20 protein on tumour cell surfaces. Furthermore, in a CDC assay they proved the biological activity of the induced rituximab-like antibodies. None of the peptides showed sequence homology to CD20 in database alignments confirming that conformational epitopes play an important role also in this type of anti-cancer therapy [115].

7.2.5 *CEACAM-5*

Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM-5) is a poorly immunogenic glycoprotein and an important tumour marker, especially for colorectal cancer. It is expressed at up to 100 times higher levels in malignant tumour tissue samples compared with healthy tissues. A biopanning performed with the monoclonal anti-CEA antibody Col-1 led to the generation of a CEA mimotope which was synthesized as an octameric MAM and further studied in a mouse model. When BALB/c mice were prophylactically immunized with the MAM before subcutaneously receiving transplants of a tumour cell line overexpressing CEA, Meth-A/CEA, a specific and significant inhibition of tumour growth could be observed [82].

7.3 Mimotopes in allergooncology

The assumption on an inverse association between allergy and IgE levels and cancer derives from epidemiological data. Among other reports, a cohort study of 1,102,247 US men and women suggested a significant inverse association between a history of hay fever, and asthma and cancer mortality [116]. Engineered anti-tumour IgE antibodies have a high cytotoxic capacity due to interaction with potent effector cells. However, natural IgE has also been described in squamous cell carcinoma of the head and neck [117] and in pancreatic cancer where its cytotoxic potential was demonstrated also [118]. The mechanisms of anti-tumour IgEs have already been studied in detail in several previous experiments [119-121], among them ADCC and antibody-dependent cell-mediated phagocytosis (ADCP) [122] seem to be most important. The IgE subclass has several advantages compared with IgG: it is bound with high affinity to receptors on its effector cells, the half-life of IgE in the periphery is longer than that of IgG and the effector cells expressing IgE receptors have been described within and around the tumour tissue [123]. Therefore, IgE antibodies directed against a tumour-associated antigen (TAA) could specifically trigger an immediate local effector cell response against the tumour cells [122].

Consequently, also the threat of systemic anaphylaxis has to be taken into consideration. However, in the presence of natural anti-tumour IgE antibodies (see above) no anaphylactic events were observed. This may be due to the fact that circulating TAAs which were shed from the tumour cell membrane are monovalent and thus not able to crosslink cell bound IgE. In contrast, overexpressed TAAs on the tumour membrane can crosslink IgE and release tumouricidic mediators from effector cells exactly at the site where they are needed.

To establish an active vaccination for the directed induction of tumour-specific IgE antibodies the above mentioned HER-2 mimotope [108] was applied orally in connection with anti-ulcer medication. The rational for this derives from the observation that IgE antibodies are preferentially induced when peptic digestion is hindered under anti-acidic treatment [124]. As expected, BALB/c mice immunized with the HER-2 mimotope via the oral route under anti-acidic treatment developed IgE antibodies cross-reactive with the original antigen HER-2. Moreover, the HER-2-overexpressing breast cancer cell line SKBR-3 specifically crosslinked the induced IgE in a rat basophilic leukemia (RBL) assay. Furthermore, the released mediators, potentially through TNF-α action [125], acted tumouricidal on SKBR-3 cells. The effect was highly specific, as with a HER-2-negative control cell line no cytotoxicity was observed [126]. Thus, depending on the route and adjuvant, mimotopes may be used for a directed induction of anti-tumour IgE antibodies.

8. Conclusion

Epitope specificity can of decisive importance in the development of vaccines, which are primarily based on a humoral immune response. In the fields of allergy and cancer, it has been demonstrated by preclinical studies that epitope mimics can be used to define B-cell epitopes on allergens/antigens of interest and, more importantly, upon immunization actively induce epitope-specific antibodies. The elicited antibodies can block the allergen–IgE interaction. Moreover, as the mimotopes do not contain allergen-specific T-cell epitopes, reactivation of inflammatory allergen-specific T-lymphocytes is not supported through a mimotope vaccine. In cancer, the mimotopes induce biological activities similarly to the original monoclonal antibody, including *in vitro* and *in vivo* growth inhibition, ADCC, CDC and ADCP. In the near future, mimotopes could be an alternative prophylactic, adjuvant and therapeutic strategy against allergy and cancer.

9. Expert opinion

Although the term 'immunotherapy' is used in allergy and cancer, the meaning is different. Cancer immunotherapy mainly focuses on passive administration of monoclonal antibodies directed against a defined part of a tumour antigen, whereas generally in allergy, immunotherapy means the injection of whole-allergen extracts inducing allergen-specific non-responsiveness. A principle of SIT in allergy for down-regulation the allergic hyper-responsiveness is the induction of blocking IgG antibodies, which should optimally be directed against IgE epitopes for successfully interfering with allergen–IgE interactions. Instead of whole allergen molecules, or even whole allergen extracts, it might be sufficient to perform immunotherapy with a subunit vaccine, such as mimotopes of B-cell epitope on the allergen.

The early trials using murine monoclonal antibodies for cancer therapy were accompanied by human anti-mouse antibody (HAMA) immune responses and relatively low efficacy due to the lack of effector functions of murine antibodies in concert with human effector cells [127]. Possibly connected with this problem at that time, much attention was rather given on the cytotoxic T-cell-dependent immunity. However, these therapies have limitations as tumour cells have developed escape strategies to avoid T-cell responses, such as downregulation of HLA [128,129]. Moreover, the applicability of T-cell-based peptide vaccines is restricted to the patient's HLA haplotype.

In the meantime, antibody immunotherapies have, due to technical advances, made considerable progress: currently chimeric and humanized monoclonal antibodies with minimal antigenicity are applied as passive immunotherapeutics, mostly in combination with chemotherapy. A major advantage of passive immunotherapy is that it can be turned off at any time point. Disadvantages are side effects (most often delayed-type hypersensitivity infusion reactions mostly due to the high antibody load leading to immune complex formation [130]), but also the high costs. To offer an alternative choice and to improve quality of life for the patients during the course of tumour disease, vaccination should be considered.

With mimotope vaccines, complete-self-antibodies with the desired epitope specificity can be induced representing a continuously available, polyclonal antibody response. Although the anti-mimotope antibodies are dominated by a certain subclass depending on the route, formulation and cytokine environment of the vaccine, the overall immune response is constituted by several isotypes mediating several immune effector mechanisms. So far, promising animal studies are available, and preclinical and clinical studies are on the way. We suggest that minimal residual disease or prophylactic approaches in high-risk patients will be the favourable setting. These studies may break the ground for active tumour immunotherapy according to the successful concept of vaccines against infectious diseases.

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Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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CHAPTER II

Aims

The overall aim of this study was to develop alternative therapeutic strategies for HER-2 overexpressing tumours based on IgE antibodies. HER-2 was chosen as tumour target because it is overexpressed in several human tumours, which is associated with a poor prognosis and an aggressive course of disease. The state of the art therapy for HER-2 overexpressing cancers is trastuzumab, an IgG1 monoclonal antibody. Although trastuzumab proved to be highly effective in clinical use, there are still some problems that have to be solved, concerning the *in vivo* stability, the tissue penetration and distribution, the recruitment of effector cells, the half-life and the costs of these therapies. Concerning these drawbacks an antibody of the IgE subclass with the same specificity would have several advantages.

Therefore, we aimed to investigate the role of IgE in addition to its well-known function in helminth infections and allergy. Considering the high capacity of IgE for mediating disadvantageous but potent inflammatory reactions, we were interested in the possible function of IgE in tumour surveillance. By specifically targeting IgE to tumour antigens in an active or passive approach, these reactions could be harnessed and directed towards malignant cells.

The first aim was to establish an oral immunization regime in a murine model to actively induce antigen-specific IgE antibodies (CHAPTER III). For this purpose, we applied ovalbumin, the major egg white allergen, under concomitant anti-acid medication and investigated the subsequent antibody response. By elevating the gastric pH, normally digestion-labile proteins remain stable and lead to the formation of antigen-specific IgE. Sera of the BALB/c mice were screened for allergen-specific antibody titres and their according

isotypes. The biological activity of IgE was tested in RBL assays and the clinical relevance was investigated by skin prick tests.

As our second and major aim was to improve the drawbacks of trastuzumab mentioned above, we focussed on the active induction of tumour-specific IgE via mimotope approach (CHAPTER IV) and on the generation of a trastuzumab-like antibody of the IgE subclass for passive immunotherapy (CHAPTER V).

The methodology and knowledge from our food allergy study was applied in an anti-cancer study which is presented in CHAPTER IV. Here, our aim was to use epitope mimics (mimotopes) of HER-2 for vaccination purposes to induce trastuzumab-like antibodies in an active manner. Feeding mice with the mimotope, under the same conditions as described in CHAPTER III, result in the induction of anti-HER-2 IgE antibodies of trastuzumab specificity and similar biological function. Importantly, we aimed to test the tumouricidic activity of the induced HER-2-specific IgEs in a cytotoxicity assay using HER-2 overexpressing cells as targets.

However, for detailed experimental comparison of anti HER-2 IgE versus IgG, we considered it important to have defined tools in our hands. Therefore, we decided to generate a trastuzumab-like IgE. We aimed to clone the epsilon part in place of the constant gamma part of the original trastuzumab (CHAPTER V). The newly engineered IgE was assessed in comparison with original trastuzumab in respect of i) binding characteristics to HER-2 and its receptors and ii) their respective killing capacities against HER-2 overexpressing tumour cells (ADCC and ADCP).

CHAPTER III

Dose-dependent food allergy induction against ovalbumin under acid-suppression: A murine food allergy model

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Dose-dependent food allergy induction against ovalbumin under acid-suppression: A murine food allergy model

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ABSTRACT

Background: Animal models are essential for analyzing the allergenic potential of food proteins and for investigating mechanisms underlying food allergy. Based on previous studies revealing acid-suppression medication as risk factor for food allergy induction, we aimed to establish a mouse model mimicking the natural route of sensitization in patients.
Methods: The effect of acid-suppressing medication on murine gastric pH was assessed by intragastric pH measurements after two injections of a proton pump inhibitor (PPI). To investigate dose-dependency, mice were fed different concentrations of ovalbumin (OVA; 0.2, 0.5, 1.0, 2.5 or 5.0 mg) either with or without anti-ulcer medication. Additionally, different routes of exposure (i.p. vs. oral) were compared in a second immunization experiment. Sera were screened for OVA-specific antibody titers (IgG1, IgG2a and IgE) in ELISA and RBL assay. Clinical reactivity was evaluated by measuring rectal temperature after oral challenge and by type I skin tests.
Results: Two intravenous injections of PPI significantly elevated the gastric pH from 2.97 to 5.3. Only oral immunization with 0.2 mg OVA under anti-acid medication rendered elevated IgG1, IgG2a and IgE titers compared to all other concentrations. Protein feeding alone altered antibody titers only marginally. Even though also i.p. immunizations induced high levels of specific IgE, only oral immunizations under anti-acids induced anaphylactic reactions evidenced by a significant decrease of body temperature.
Conclusion: Only low-dosage ovalbumin feedings under anti-acid medication resulted in IgE mediated food allergy. Based on this knowledge we have established a suitable food allergy model for further investigations of food adverse reactions.

1. Introduction

Today, population studies indicate a rising prevalence of food allergies in western societies [1,2]. Consequently there is a growing need to investigate in-depth mechanisms and risk factors leading to sensitization towards food compounds. For this purpose, animal food allergy models including rodent, swine and dog models are used and discussed to have the potential to mimic the human disease process [3]. Additionally, in the 2001 joint FAO/WHO meeting animal models were suggested as suitable tools for the analysis of the allergenic potential of novel dietary compounds [4], which should be further evaluated for structural homologies or sequence similarities with known allergens, included in allergy testing and should be assessed for their digestion stability [5].

Despite significant differences between human and mouse immunology [6], mice are the preferentially used organism for food allergy models. A wide range of inbred strains have been characterized as being either high or low IgE responder animals [7]. The easy handling and the possibility to include a larger number of animals per group account for the widespread use. Important for these studies are immunological similarities between mice and humans as both produce IgE antibodies, which bind to the high affinity receptor FcεRI being expressed on mast cells and basophils.

Abbreviations: OVA, ovalbumin; PPI, proton pump inhibitors; SGF, simulated gastric fluid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; i.v., intravenous; i.p., intraperitoneal; i.g., intragastric; $Al(OH)_3$, aluminum hydroxide; ELISA, enzyme-linked immunosorbent assay; TBST, Tris buffered saline with Tween-20; DMP, dried milk powder; TMB, tetramethylbenzidine; RBL, rat basophil leukaemia; 4-MUG, 4-methylumbelliferyl β-D-galactopyranoside; HRP, horseradish peroxidase.

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In both species gastrointestinal symptoms depend on synthesis and secretion of serotonin and platelet-activating factor [8].

Recently, we have developed a novel oral immunization protocol in BALB/c mice inducing an IgE mediated response by protein feedings under concomitant anti-acid medication [9–11]. In these studies, the normal function of the stomach representing a gatekeeper against food allergens has been determined as an important factor for preventing food allergies [12].

The relevance of this mechanism was demonstrated both in the murine system [9–11] as well as for humans, as 25% of gastroenterological patients being treated with acid-suppression medication for a 3-month period due to dyspeptic disorders revealed formation of specific IgE antibodies and sensitization towards dietary compounds [9,13].

Based on this knowledge we aimed to establish a true mouse food allergy model exploiting the oral route for immunizations under concomitant acid-suppression treatment. The major egg white allergen ovalbumin (OVA), a 45 kDa protein constituting about 54% of all egg white proteins, was chosen as a model antigen due to its wide use in allergy research to enable a better comparison with previously established food allergy models. Moreover, we wanted to standardize our immunization regimen addressing the effect of dose-dependency on food allergy induction.

2. Materials and methods

2.1. The model allergen ovalbumin

Lyophilized OVA (Sigma, Vienna, Austria, 98% purity) was used for all experiments. Simulated gastric fluid (SGF) was prepared with a pharmaceutical enzyme tablet (Enzynorm® forte, Pharmaselect Handels GmbH, Vienna, Austria) as previously described [14] with slight modifications: one tablet was dissolved in 100 μL 0.9% sterile sodium chloride at pH 2.0 or pH 5.0. For digestion, 500 μL of SGF was incubated with 500 μg OVA. The digestion was quenched with 0.1 N NaOH after 1, 5, 15, 60 and 120 min. The effect of incubation with SGF on protein integrity was evaluated in SDS-PAGE using Coomassie brilliant blue staining [15].

2.2. Animals

Four to six weeks old female BALB/c mice were purchased from the Institute of Laboratory Animal Science and Genetics, Medical University of Vienna, Austria. All experiments were performed according to European Community rules of animal care (permission numbers GZ 66.009/0039-BrGT/2005 and GZ 66.009/0170-BrGT/2006 of the Austrian Federal Ministry of Science and Research).

2.3. Intragastric pH measurements after acid-suppression

Six weeks old female BALB/c mice were divided into two groups ($n = 10$). After overnight fasting mice were either left untreated or were injected intravenously (i.v.) with the proton pump inhibitor omeprazole (PPI, Losec®, AstraZeneca GmbH, Wedel, Germany; 116 μg omeprazole diluted in 0.9% sodium chloride), which was followed by a second i.v. injection after 1 h. After 15 min, mice were sacrificed and the stomach was immediately removed and perfused with 150 μL sterile sodium chloride. The pH of this washing fluid was measured using a pH microelectrode.

2.4. Immunization protocol

For investigating the effect of antigen dosage, animals were divided into 10 groups ($n = 5$ each). Based on the data generated by intragastric pH measurements groups 1–5 were medicated intravenously with the proton pump inhibitor for 3 days (on days 1–3, 16–18 and 29–31). On days 2–3, 17–18 and 30–31 mice were immunized orally with different concentrations of OVA (0.2, 0.5, 1.0, 2.5, 5.0 mg per mouse) mixed with 2 mg sucralfate (Ulcogant®, Merck) 15 min after a repeated i.v. injection of the PPI. Groups 6–10 were fed the allergen at the different concentrations without PPI on the respective days. Blood samples were taken on days 0, 15, 28 and 42.

To compare different routes of exposure, the immunization experiments were repeated with four groups of animals ($n = 10$ each). Group A was immunized intraperitoneally (i.p.) with 2 μg OVA adsorbed to 2% aluminum hydroxide solution (1.3 μg $Al(OH)_3$). Group B (0.2 mg OVA i.g. under acid-suppressing medication) and Group C (0.2 mg OVA i.g.) were immunized following the same protocol with the previously selected concentration. The negative control Group D remained naïve.

All immunizations were performed in two independent sets of experiments.

2.5. Evaluation of OVA-specific antibodies in ELISA, RBL-assay and dot blot experiments

Murine sera were screened for OVA-specific antibody subclasses (IgG1, IgG2a) in an enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Maxisorp, NUNC, Roskild, Denmark) were coated with 1 μg OVA per well. After blocking with TBST (Tris buffered saline with Tween-20) with 1% dried milk powder (DMP), mouse sera diluted 1:100 for IgG1 and IgG2a in TBST/0.1% DMP were incubated overnight at 4 °C. Bound antibodies were detected using rat anti-mouse IgG1 and IgG2a (BD Biosciences, Franklin Lakes, NJ; 1:500) followed by a peroxidase labeled goat anti-rat IgG (Amersham, Buckinhamshire, UK, diluted 1:1000). For detection, TMB (tetramethylbenzidine, BD Bioscience, Vienna, Austria) was added for 15 min and the reaction was stopped with 1.8 M H_2SO_4. The color reaction was measured at 450–630 nm. Antibody concentrations were calculated according to standard dilution series after subtracting levels detected in pre-immune sera as background values.

To evaluate biologically active OVA-specific IgE, a rat basophil leukemia cell assay (RBL-assay) was performed [16]. RBL-2H3 cells, exclusively expressing the high affinity IgE receptor FcεRI [17], were passively sensitized with murine sera (dilution 1:10) and incubated at 37 °C for 1 h. After washing, 10 μg/mL OVA were added to the appropriate wells. The induced β-hexosaminidase release was detected using 4-methylumbelliferyl β-D-galactopyranoside (4-MUG, Sigma, Vienna, Austria) and the fluorescence was measured at 360–465 nm. Calculation was made by correlating measured values with β-hexosaminidase release of cells lysed with triton-x (Sigma), which was set 100%.

In order to evaluate the binding capacity of OVA-specific IgE antibodies to undigested antigen and to OVA digested for 120 min either at pH 2 or pH 5 conditions, dot blot experiments were performed. Therefore, 1 μg of the appropriate antigen, either OVA or as control α-casein, were dotted onto nitrocellulose membranes (Life Sciences Bio Trace, Pall Corporation, Vienna Austria). After blocking with 1% DMP in TBS 0.1% Tween blot stripes were incubated with serum samples taken before the first and after the last immunization (1:500 diluted in TBS 0.1% Tween) for 2 h. After extensive washing, first rat anti-mouse IgE (1:1000) and after 1 h peroxidase labeled goat anti-rat IgG antibodies (1:3000) were added for detection. The blot stripes were developed by luminescence reaction using the ECL kit (Amersham).

2.6. In vivo read-out by rectal temperature measurement after oral antigen challenge and type I skin testing

On day 49, animals were fed with 2 mg OVA in PBS (oral challenge) for evaluating anaphylactic reactions. Rectal temperature was measured before and 15 min after oral challenge with a thermometer (Thermalert TH5, Clifton, NJ).

To evaluate the in vivo relevance of the induced OVA-specific antibodies, type I skin tests were performed on day 50. 100 μL of 0.5% Evans Blue (w/v) were applied intravenously. 0.6 μg of OVA and the control antigen α-casein, both diluted in sterile sodium chloride were injected intradermally into the shaved abdominal skin. The mast cell degranulation compound 48/80 (Sigma, 20 μg) was used as positive and sterile sodium chloride as negative control. After 20 min, animals were sacrificed and the color reaction was evaluated on the inside of the abdominal skin.

2.7. Cytokine determination

As previously described [11], spleen cells were removed under sterile conditions, minced and passed through sterile nylon filters (cell strainer 40 μm Nylon). Erythrocytes were lysed in 0.75% ammonium chloride (3 mL) for exactly 4 min. After stopping the reaction cells were washed for 3 times in medium. Cell number was determined in a Coulter Counter. For spleen cell stimulation, 4×10^5 cells per well were incubated at 37 °C in triplicates with OVA (20 μg/mL), medium (as negative control) or concanavalin A (as positive control; 50 μg/mL) for 4 days. Thereafter, 100 μL spleen cell supernatants were withdrawn for evaluation of cytokine levels and were screened for IL-5 and IFN-γ using a capture ELISA (eBioscience Ready-set-go! IL-5 or IFN-γ Femto HS, San Diego, CA) following the manufacturer's instructions. In short, after coating the appropriate capture antibody, plates were blocked with assay diluent for 1 h. Either standard (recombinant mouse IL-5 or IFN-γ) or mouse spleen cell supernatants were incubated for 2 h. After extensive washing, a biotin-labeled detection antibody was added. After 1 h incubation with avidin-HRP, the reaction was developed using a substrate solution (TMB-solution) for 15 min and the absorbance was read at 450–570 nm. Comparisons were performed by calculating concentrations according to a standard curve after subtracting medium values as background levels.

2.8. Data analysis

Antibody titers, RBL-assay and cytokine results were statistically compared using the non-parametric Mann–Whitney *U* test. pH measurements and temperature results were compared using the two-tailed Student's *t*-test with the SPSS 14.0 program. A *P* value <.05 was considered statistically significant.

3. Results

3.1. Digestion stability of OVA to simulated gastric fluid

In line with previously published data [18], SGF digestion of OVA using a pharmaceutical enzyme tablet revealed that OVA proteins were degraded within 60 min of gastric digestion at pH 2.0 (Fig. 1A). However, by increasing the pH conditions to pH 5.0 the protein bands remained stable up to 120 min (Fig. 1B), representing the average gastric transit time.

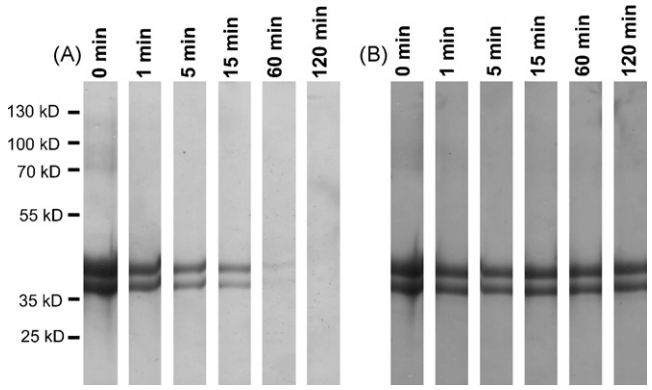

Fig. 1. Impaired OVA digestion at elevated pH levels of SGF. OVA (lane 1) was incubated with pepsin for 1, 5, 15, 60 and 120 min either at pH 2 (panel A) or pH 5 (panel B). All proteins were degraded after 60 min of digestion at pH 2. However, by increasing the pH of the simulated gastric fluid to pH 5 all proteins remain stable up to 120 min.

3.2. Two injections with a proton pump inhibitor significantly increases the gastric pH

To evaluate the effect of PPI medication on the murine gastric environment, we performed intragastric pH measurements after two i.v. injections of the PPI omeprazole. As depicted in Fig. 2, the gastric pH levels increased significantly from pH 2.97 in the untreated animals (group A) to pH 5.30 measured 15 min after the second PPI injection (group B).

3.3. OVA feeding under acid-suppression induces antigen-specific antibodies in a dose-dependent manner

Based on the results from intragastric pH measurements, we have medicated mice intravenously with the PPI on 3 consecutive days for 3 times and further immunized orally with OVA (group 1–5). For an additional Th2 biasing effect [19], the aluminum containing anti-acid sucralfate was added to the solution, which was fed the animals. To further address the question of dose-dependency, we have used different OVA concentrations (0.2, 0.5, 1.0, 2.5 and 5.0 mg per mouse) for the feedings. As controls,

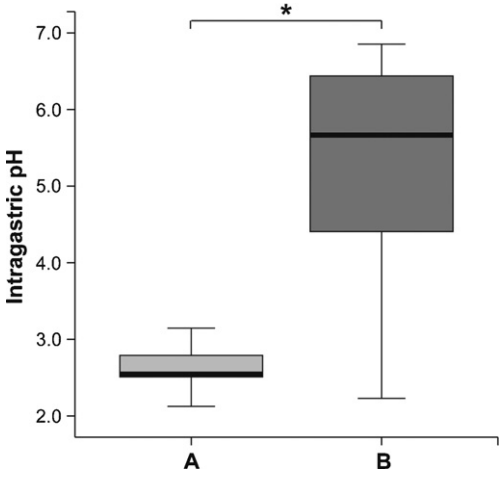

Fig. 2. Repeated anti-acid medication elevated intragastric pH levels. Compared to naïve animals (group A), mice being medicated twice intravenously with the proton pump inhibitor (group B) showed a significant increase of intragastric pH already 15 min after the second injection. The boxes represent the inner quartiles value range with the median indicated as black line. Brackets indicate the statistically significant difference of intragastric pH (*$P < .05$).

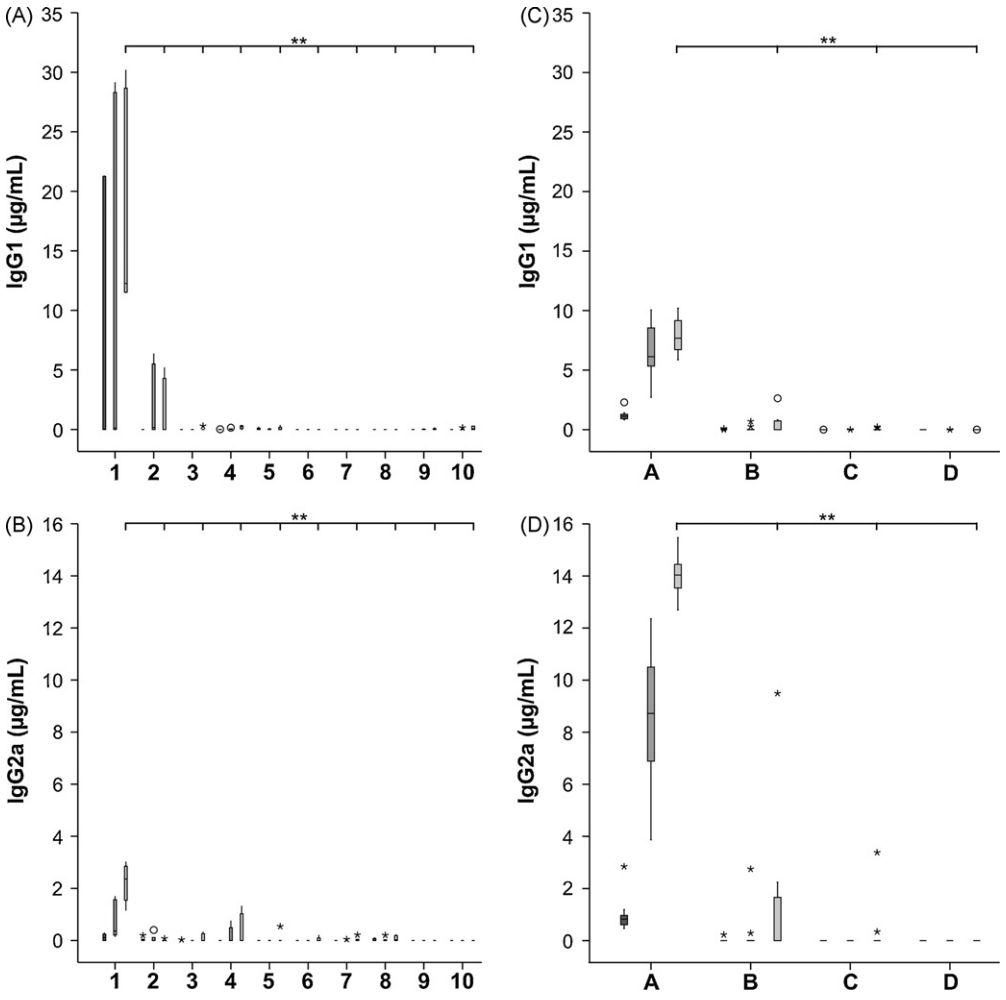

Fig. 3. OVA-specific antibody induction in acid-suppressed mice. Acid-suppressed animals (1–5) or BALB/c mice without anti-acid medication (group 6–7) were fed different concentrations of OVA (0.2 mg: groups 1 and 6; 0.5 mg: groups 2 and 7; 1.0 mg: groups 3 and 8; 2.5 mg: groups 4 and 9; 5.0 mg OVA: groups 5 and 10). Only acid-suppressed mice receiving 0.2 mg OVA showed significantly increased levels of IgG1 and IgG2a (panel A and B). Comparing the effect of i.p. immunization with oral feeding (panel C and D) higher levels of IgG1 and IgG2a were observed in the positive controls (group A; OVA i.p.) and to a lesser extent in the acid-suppressed animals (group B; OVA i.g. + PPI). No changes in antibody titers were observed in mice fed the protein alone (group C) and in the naïve animals (group D). The boxes (first immune serum, dark grey; second immune serum, middle grey; third immune serum, light grey) represent the inner quartiles value range with the median indicated. Sera with signals showing more than 1.5-fold interquartile range deviation from the end of the box were defined as outliers and marked as circles. Sera with titers lying more than 3-fold interquartile range away were defined as extremes and marked with asterisks. Brackets indicate statistically significant differences of antibody concentrations (**$P < .01$).

mice were fed the respective OVA concentration without acid-suppression (group 6–10). In the medicated animals, the IgG1 and IgG2a levels were significantly increased only in group 1 being fed 0.2 mg OVA under acid-suppression (Fig. 3A and B). Interestingly, immunizations with all other allergen concentrations (0.5, 1.0, 2.5 or 5.0 mg per gavage) did not elevate antibody titers even under concomitant anti-acid therapy. In the second set of experiments, comparing different routes of immunization, especially the positive control group A (OVA i.p.) developed high levels of both IgG subtypes and to a lesser extent also the acid-suppressed animals of group B. Nevertheless, differences in IgG1 levels were observed between groups 1 and B in the two sets of experiments. Animals being fed the OVA proteins alone (Group C) and naïve mice (Group D) showed no altered antibody levels (Fig. 3C and D).

In a RBL assay, murine sera taken after the last immunization were assessed for functional antigen-specific IgE antibodies. Only the acid-suppressed animals being fed 0.2 mg OVA showed significantly increased β-hexosaminidase release compared to all other immunization concentrations (Fig. 4A). These findings were confirmed in the second set of experiments, where a significantly elevated β-hexosaminidase release was observed in the positive control group A and after oral immunizations with OVA under acid-suppression (Group B) (Fig. 4C).

Dot blot experiments were performed to evaluate the binding capacity of specific IgE antibodies to undigested OVA and further to OVA digested either at pH 2.0 or pH 5.0. In accordance with the RBL assay results, only IgE of the animals being fed 0.2 mg OVA under acid-suppression and the mice being injected OVA i.p. bound specifically to undigested OVA and to the protein being digested at elevated pH conditions. Only background reactivity was observed with proteins digested at pH 2 for 120 min. Neither sera obtained from other groups (Fig. 4B and D) nor preimmune sera (data not shown) revealed binding reactivity with the dotted OVA samples.

3.4. Decreased body temperature and positive skin testing reveal food allergy in animals

To assess shock symptoms causing blood centralization and, therefore, a decline of body temperature, the rectal temperature was measured in immunized animals before and after oral provocation with OVA. Only the animals which were immunized orally with 0.2 mg OVA under anti-acid medication revealed a signifi-

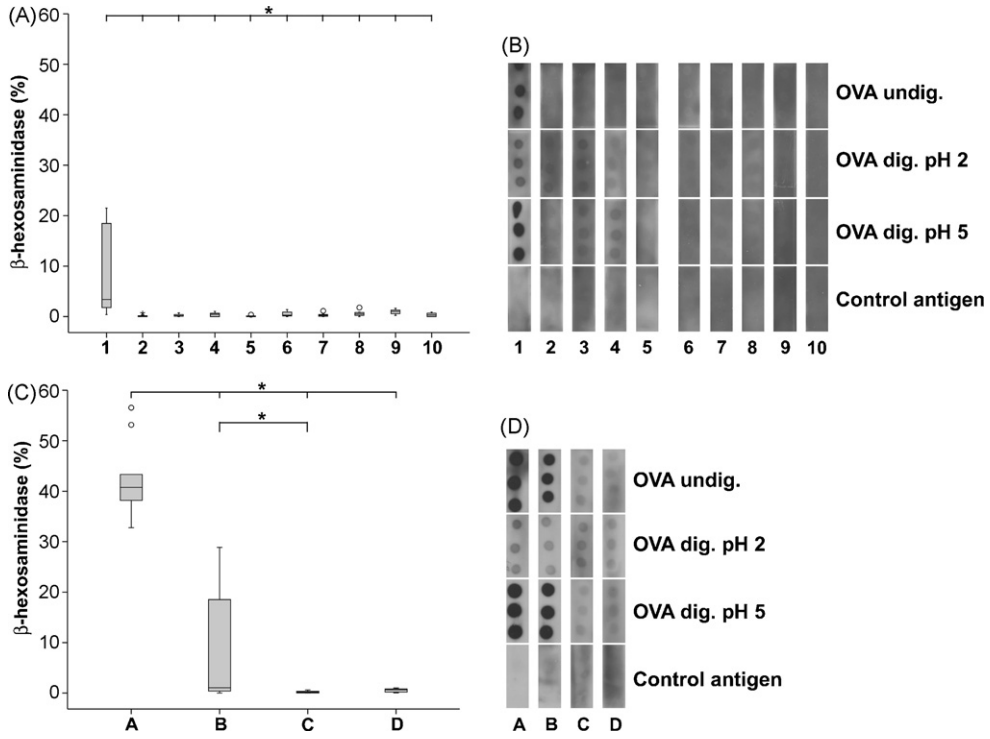

Fig. 4. Biologically active IgE was induced in mice under anti-ulcer medication. Gavages of 0.2 mg OVA under anti-acid treatment (lane 1) induced significantly higher IgE levels compared to other groups (lanes 2–10) (panel A). Intraperitoneal immunizations (group A) and oral injection under acid-suppression (group B) revealed significantly higher values of specific IgE compared to feeding OVA alone (group C) and naïve animals (group D) (panel C). In dot blot experiments (panels B and D) only the i.p. immunized mice (group A) and the acid-suppressed animals being fed 0.2 mg OVA (lanes 1 and B) revealed IgE binding capacity to undigested OVA and OVA digested at pH 5.0. In all other groups only background reactivity was observed. The IgE binding was diminished when testing with OVA digested at pH 2.0. Representative dot blots are shown.

cant decline in body temperature 15 min after the oral challenge ($P = 0.046$), whereas all other groups receiving the antigen via the oral route showed only a marginal decrease or even an increase of body temperature (data not shown). These data were confirmed in the second set of experiments. Despite the high levels of IgE antibodies detected in the sera of animals of group A (OVA i.p.) a significant decrease of body temperature was detected only in group B immunized with OVA under anti-ulcer medication (Fig. 5).

To evaluate the in vivo relevance of detected antibodies, type I skin tests were performed in the immunized animals. Only animals immunized with 0.2 mg OVA under acid-suppression showed positive skin reactions to OVA, whereas no other oral immunization protocol revealed any reactivity to the tested allergens. Intraperitoneal immunization resulted in positive skin reactions only to OVA and to the positive control (compound 48/80), whereas naïve animals did not develop any skin reactivity except to the positive control antigen (Fig. 6).

3.5. Antigen feeding under gastric acid-suppression induces Th2 cytokine production

In order to evaluate a possible Th2 switch induced by oral immunization under anti-ulcer medication, spleen cell supernatants of mice from the second experiment were screened for IL-5 as a Th2 marker, and IFN-γ as a Th1 marker. Compared to the animals being fed OVA alone, the i.p. immunized mice developed a 20-fold increase and the acid-suppressed animals revealed a 12-fold increase of mean IL-5 levels, although differences were not statistically significant. The IL-5 levels of naïve animals were below detection limits. In contrast, the IFN-γ levels of the animals being injected OVA i.p. and of the mice being medicated with anti-ulcer

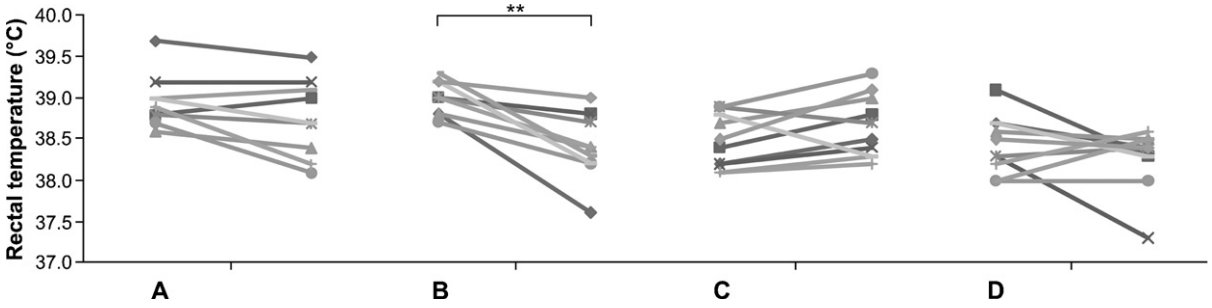

Fig. 5. Acid-suppressed animals revealed a significant decrease of body temperature after oral OVA challenge. Compared to the positive control (OVA i.p., group A), the animals fed OVA alone (group C) and the naïve animals (group D), only group B (0.2 mg OVA i.g. with anti-acids) revealed a significant decrease of rectal temperature. Brackets indicate the statistically significant differences of rectal temperature before and 15 min after oral OVA challenge (**$P < .01$) within group B.

Table 1
Cytokine levels measured in OVA stimulated spleen cell supernatants

	Group A OVA i.p.	Group B i.g. + antacid	Group C OVA i.g.	Group D naïve
IL-5				
Mean	69.47	40.62	3.34	0
95% confidence interval	12.30–126.65	0–101.08	0–8.51	0–0
IFN-γ				
Mean	109.34	140.43	67.37	68.64
95% confidence interval	0–271.87	0–348.31	0–177.17	0–195.49

Cytokine concentrations are given as mean and 95% confidence interval in pg/mL.

drugs were twofold increased compared to animals being fed the antigen alone and to naïve animals (Table 1).

4. Discussion

Recently, we were able to show that acid-suppression treatment interfering with gastric digestion of proteins in murine as well as human studies leads to IgE induction and food allergy [9–11,13]. The intake of anti-acid medication, e.g. sucralfate or proton pump inhibitors, results in an elevation of the gastric pH. Under these circumstances, normally digestion-labile proteins remain stable and their conformation intact, which enhances the possibility for de novo sensitization and formation of antigen-specific IgE [12].

The aim of the current study was to experimentally investigate underlying mechanisms and to compare our murine model of food allergy with other immunization protocols and further to evaluate the dose-dependency of antigen under acid-suppression.

For this purpose, we have chosen OVA as a model antigen. Even though OVA is generally accepted as a typical class 1 allergen being of high stability to thermal and enzymatic degradation [20], it was previously reported to be degraded within 60 min in simulated gastric fluid experiments [18]. Indeed, also in our SGF assays using a pharmaceutical gastric enzyme tablet these data could be confirmed. However, if physiological low pH conditions (pH 2.0) were changed to pH 5.0 which is found after 5 days of treatment with proton pump inhibitors [21] OVA proteins remain stable up to 120 min representing the normal gastric transit time. Interestingly, apart from protein fragmentation, gastric digestion was additionally described to substantially change the immunological behavior of OVA by altering its biochemical features [22] and was shown to have impact on antigen presentation [23]. Therefore, we suggest OVA as suitable and highly relevant model food protein for our immunization protocol.

In a first experiment we wanted to confirm the acid-suppressing effect of PPIs on the murine gastric environment. Already two cycles of i.v. PPI injections significantly elevated the intragastric pH of BALB/c mice from 2.97 to 5.30. Thus, having a similar effect as on the human pH levels [21] we have used repeated injections of PPI in our immunization protocol. We additionally combined it with sucralfate, as aluminum-compounds are known to establish a pronounced Th2 response [24], which was recently also evidenced for sucralfate [19,25]. As both drugs were previously reported to induce a Th2 biasing effect only in combination with the respective allergen [9,11,19], we expected a profound Th2 induction by this immunization protocol.

In a first set of murine immunizations we addressed the question of dose-dependency on allergy induction. It has been previously reported that high doses of allergens are known to induce tolerance rather than sensitization [26]. Even though results might be influenced by chosen mouse strains, it was demonstrated that feedings of high concentrations of OVA (20–100 mg) suppressed antigen-specific IgG1, IgG2a and IgE antibody levels [27]. However, it was further described that rather low doses of OVA (0.25 mg) may also render a reduction in IgE levels [28]. Furthermore, low doses of OVA feedings (0.4–0.5 mg) did not induce specific IgE production when given without adjuvant [29]. In line with these results, feedings of OVA alone did not induce sensitization at any concentration. Whereas recent data revealed feedings of low con-

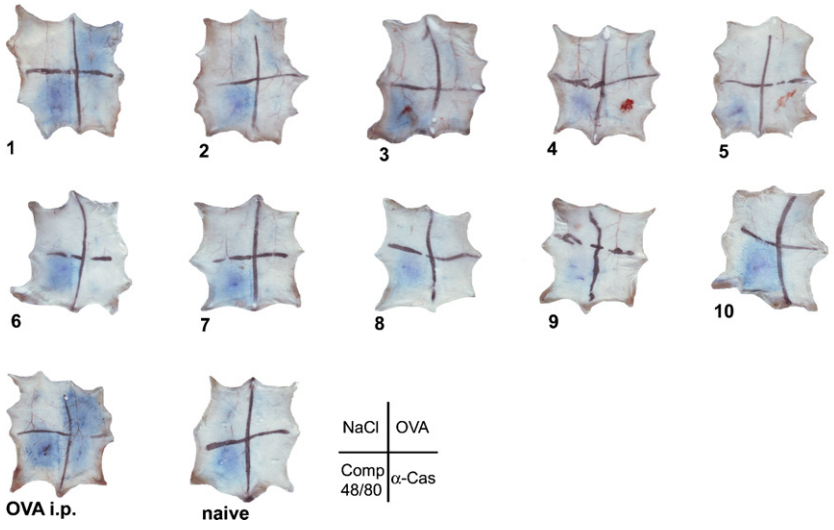

Fig. 6. Feedings of OVA under acid-suppression induced positive type I skin tests. Group 1 (0.2 mg OVA i.g. with anti-acids) and the i.p. immunized animals revealed positive skin reactions to OVA (top right) and the positive control compound 48/80 (bottom left), but no reactions to sodium chloride (top left) and the control antigen (α-casein). No skin reactions were found in the acid-suppressed animals fed OVA with other concentrations (2–5), in the mice fed the protein alone (6–10) or in the naïve animals. Representative skin tests are shown. The skin test injection scheme is depicted in the right corner.

centrations of OVA (0.2 and 1 mg) with cholera toxin as adjuvant preferentially induced antigen-specific IgG1 [30], we could show that gavages of the lowest dose of OVA (0.2 mg) under concomitant acid-suppression resulted in significantly higher formation of antigen-specific IgG1, IgG2a and IgE compared to exclusive OVA feedings. Furthermore, only this immunization protocol with the lowest antigen dose revealed a significant drop of body temperature after oral OVA challenge.

We applied the concentration of 0.2 mg OVA in a second set of murine immunizations for comparing different routes of immunization. Therefore, we included a positive control of i.p. immunization and compared the induced antibodies with the levels observed in naïve animals. Here we could confirm the humoral immune response upon OVA feeding under anti-acid treatment, underlined by elevated IgG1 titers and biologically functional IgE. Also the positive controls developed high levels of all antibody subclasses, which is in line with previous studies [31,32]. Comparable to this route, even subcutaneous immunizations with OVA adsorbed to aluminum hydroxide were reported to induce high levels of IgG1 and IgE [33,34]. Furthermore, in our experiments both immunization regimens, i.p. injection of OVA and feeding under acid-suppression rendered formation of the Th2 cytokine IL-5 and additionally revealed positive type I skin tests to OVA, whereas mice being fed OVA alone and naïve animals did not show skin reactions upon allergen testing.

Most importantly we observed a significant reduction of body temperature after oral antigen challenge only in the animals immunized with low dosage of OVA under concomitant acid-suppression (Fig. 5). The measurement of body temperature has since long been applied for the read-out of anaphylactic reactions in numerous studies [35–37]. Interestingly, the induction of an anaphylactic reaction upon oral antigen challenge exclusively in the acid-suppressed animals might result from the induced titers of different allergen-specific antibody isotypes. It has been hypothesized that the lack of a clinical reactivity following i.p. immunization with aluminum as an adjuvant could be due to existence of blocking antibodies of the IgA and IgG subclass [8].

Based on the current data we suggest our murine model to represent an experimental food allergy model mimicking the situation in allergic patients and confirming the dose-dependent sensitization capacity of allergens, again under acid-suppression [9,13]. Thus, we may have a valid model in our hand for further investigations of mechanisms in food allergy and for safety testing of novel dietary compounds.

Acknowledgments

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CHAPTER IV

Active induction of tumor-specific IgE antibodies by oral mimotope vaccination

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Research Article

Active Induction of Tumor-Specific IgE Antibodies by Oral Mimotope Vaccination

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Abstract

A role of IgE antibodies in cancer surveillance has been implicated for a long time. Studies dealing with IgE antibodies directly targeted to tumor antigens have shown marked anticancer effects mediated by this antibody class. Thus, the basic function of IgE antibodies may be to control tumor growth. Thus far, cancer-specific IgE has only been applied passively. Consequently, the aim of this study was to establish an active vaccination protocol to induce tumor antigen–specific IgE antibodies, and to evaluate functional properties. We previously generated epitope mimics, so-called mimotopes, for the epitope recognized by the anti-HER-2 antibody trastuzumab. Upon i.p. immunizations, IgG antibodies with trastuzumab-like properties could be elicited. In the present study, we immunized BALB/c mice via the oral route with these trastuzumab mimotopes, under simultaneous neutralization and suppression of gastric acid. As shown in preceding experiments, this feeding regimen effectively induces Th2 immune responses. Oral immunizations with trastuzumab mimotopes under hypoacidic conditions indeed resulted in the formation of IgE antibodies towards the HER-2 antigen. Moreover, anti-HER-2 IgE-sensitized effector cells mediated SK-BR-3 target cell lysis in an antibody-dependent cytotoxicity assay. We conclude that directed and epitope-specific induction of IgE against tumor antigens is feasible with an oral mimotope vaccination regimen, and that these antibodies mediate anticancer effects. [Cancer Res 2007;67(7):3406–11]

Introduction

The concept of immune surveillance was first proposed almost a century ago by Paul Ehrlich (1), and as immunosurveillance hypothesis against malignant disease, was again put forward in 1957 by Francis McFarlane Burnet (2). It suggests that the immune system is continuously searching for and destroying tumor cells as they arise. Evidence for the immune surveillance theory in humans derives from the observation that those who are immunosuppressed exhibit a higher incidence of malignancy (3).

Early on, it was suggested that those with a history of allergy, in particular atopic disorders, may possess an enhanced capacity for immune surveillance. Beginning in 1953, with the study by Logan and Saker (4), and around the 1960s (5–7) and early 1970s (refs. 8–12, reviewed in ref. 13), the first observations were published that allergy could be inversely associated with cancer development. For several decades, epidemiologic studies have tried to correlate the incidence of allergic disease and diverse tumors, however, the results were mostly controversial and depended on the type of malignancy. In 2005, Turner et al. published the first large prospective cohort study to examine the relationship between atopic disease and cancer mortality, including 1,102,247 U.S. men and women for 18 years of follow-up (14). In this study, they found significantly lowered risks of ~10% for overall cancer mortality and 20% for colorectal cancer mortality among persons with a history of both asthma and hay fever. A history of hay fever alone was associated with a significantly lowered risk of pancreatic cancer mortality, and a history of asthma only was associated with a lowered risk of leukemia mortality.

Also in 2005, Wang and Diepgen published a review of the epidemiologic studies after 1985 (15). Their general conclusion was that allergy is associated with a reduced risk for cancer. This stands especially for colon cancer, pancreatic cancer, childhood leukemia, and brain tumors.

In 2006, the Turner group published another overview of the epidemiologic evidence by using the MEDLINE database since 1966 (16). Again, strong inverse associations were reported for allergy and pancreatic cancer and glioma, whereas lung cancer was positively associated with asthma.

This evidence relates to conditions with overall elevated IgE levels. Only a small number of studies have thus far investigated the effects of tumor-specific IgE (17–21). All of them came to the conclusion that IgE directly targeted to cancer antigens is extremely efficient in eliciting antitumor effects. In 1991, Nagy et al. developed a murine monoclonal IgE against the murine mammary tumor virus. It was applied for passive immunizations of mice, and prevented the development of s.c. tumors in an antigen-specific fashion (17). Kershaw et al. also developed a monoclonal IgE, recognizing a colon carcinoma antigen, and were the first to prove that the antitumor effect of targeted IgE was isotype-dependent (18). In 1999, Gould et al. generated mouse-human chimeric IgG_1 and IgE monoclonal antibodies against the ovarian cancer antigen MOv18 and compared them in a severe combined immunodeficiency mouse xenograft model of ovarian carcinoma. They found that the beneficial effects of tumor-specific IgE were greater and of longer duration than those of IgG_1 (19). In a subsequent study, this group showed that IgE triggered antibody-dependent cellular cytotoxicity (ADCC) via monocytes (20). The last study dealing with IgE directly targeted to tumor antigens postulated that IgE loaded onto tumor cells could affect tumor immunogenicity by activation of the innate immune system directly at the tumor site. They used a biotin-avidin bridging strategy to target IgE to the tumor cells, and found that IgE strongly affects tumor growth *in vivo*, leading, in some cases, to complete tumor rejection and protection against subsequent tumor challenges by a mechanism involving both eosinophils and

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T cell–mediated antitumor responses. Moreover, the results showed that IgE targeted to tumor cells not only possess curative potential, but also confer long-term antitumor immunity (21).

From all these data, we believe that IgE, especially IgE targeted to tumor antigens, plays a beneficial role in antitumor immunity. Therefore, we aimed to move the approaches of passive application of tumor-specific IgE a step forward—by developing a vaccine that would induce tumor-specific IgE *in vivo*. To this end, we combined two strategies that were developed in our group during the past few years: first, an epitope-specific vaccination against the HER-2 tumor antigen, rendering antibodies with similar biological properties as the monoclonal antibody trastuzumab (22); second, an oral immunization regimen discovered in food allergy research, resulting in IgE induction (23, 24). Here, we show for the first time the active induction of tumor-specific IgE antibodies, targeting HER-2 as an important model antigen.

Materials and Methods

Cell lines and monoclonal antibody. The HER-2-positive human mammary carcinoma cell line SK-BR-3 (ATCC HTB-30) was grown in McCoy's medium (Life Technologies, Inchinnan, United Kingdom) supplemented with 10% FCS, 1% glutamine, 1% penicillin/streptomycin, and 50 μg/mL of gentamicin sulfate. The human squamous carcinoma cell line A-431 (CRL-1555; American Type Culture Collection, Manassas, VA), which is HER-2-negative, was grown in DMEM (Life Technologies) supplemented as above, and used for control purposes.

Rat basophilic leukemia RBL-2H3 cells were kindly supplied by Dr. Arnulf Hartl, Paracelsus Medical University, Salzburg, Austria. They were grown in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 4 mmol/L of L-glutamine, 2 mmol/L of sodium pyruvate, 10 mmol/L of Hepes, 100 μmol/L of 2-mercaptoethanol, and 1% penicillin/streptomycin. Trastuzumab (Herceptin), a humanized IgG_1 monoclonal antibody targeting HER-2, was purchased from Roche (Hertfordshire, United Kingdom).

Mimotope characterization and vaccine compound. Mimotopes mimicking the trastuzumab epitope on HER-2 were previously defined (22, 25). The best peptide mimic of these experiments, the 1,12-cyclic peptide C-QMWAPQWGPD-C, was manufactured synthetically (piChem, Graz, Austria) and coupled via its COOH terminus to a succinimidyl-4-(*N*-maleinimidomethyl)cyclohexane-1-carboxylate activated immunogenic carrier, keyhole limpet hemocyanin (KLH), through a linker (GPGPG) by *S*-acetyl-thio-acetate. The conformational accuracy of the coupled mimotope was verified with specific recognition by trastuzumab. As a control, the peptide 1,12-cyclic C-DGGWLSKGSW-C, which did not bind to trastuzumab, was produced and coupled in the same way.

Immunization of BALB/c mice. Six-week-old female BALB/c mice (n = 7 per group) were obtained from the Institute for Laboratory Animal Science and Genetics (University of Vienna, Vienna, Austria) and were treated according to European Community rules of animal care with permission no. 66.009/35-BrGT/P2004 from the Austrian Federal Ministry of Education, Science, and Culture.

Mice were immunized according to the oral immunization regimen for IgE induction developed in our group (23), with some modifications. Amounts of antiulcer drugs were correlated with the dosage recommended for use in humans and adjusted for body weight and metabolism of mice. Each animal received 11.6 μg of omeprazole (Losec, AstraZeneca, Vienna, Austria) i.v. on days 1, 2, and 3 of each immunization cycle. On day 3, omeprazole was applied 2 h and 15 min before intragastric immunization. One hundred micrograms of the respective antigen were mixed with 2 mg of sucralfate (Ulcogant; Merck, Vienna, Austria) and administered intragastrically in a final volume of 100 μL. The verum group was immunized with the C-QMWAPQWGPD-C mimotope-KLH conjugate, and the control group received the carrier KLH alone. Immunizations were done on days 3, 17, 45, 59, and 87. Blood was taken from the tail vein on day 0 (preimmune serum), and on days 27, 69, and 97 (first, second, and third mouse immune serum).

Titer determination. IgE and IgG titers of sera from the immunized BALB/c mice against KLH and against the mimotope peptide were determined by incubation of serial dilutions of pooled group sera with DotBlots of KLH, the C-QMWAPQWGPD-C mimotope peptide, and the control peptide C-DGGWLSKGSW-C. In short, antigens were solubilized in PBS/20% dimethylformamide, and dotted onto nitrocellulose membrane at 1 mg/mL. Blot strips were incubated with mouse serum dilutions, and bound antibodies detected with rat anti-mouse IgG or rat anti-mouse IgE antibodies (PharMingen, San Diego, CA), that were again detected by a peroxidase-conjugated sheep anti-rat immunoglobulins (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) using the ECL chemoluminescent detection protocol (Amersham). Trastuzumab was used as a positive control and detected with a peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch, West Grove, PA). Titers were given as the highest serum dilution at which antigen reactivity was still detectable in these DotBlot experiments.

β-Hexosaminidase release assay from RBL-2H3 cells. RBL-2H3 cells, which express rodent FcεRI as their only antibody receptor, were used to determine functional HER-2–specific IgE. Cells were incubated with individual third immune sera of the mimotope-immunized or the control mice to allow for binding of serum IgE to FcεRI. Loaded RBL-2H3 cells were then incubated with SK-BR-3 cells as a source of natural HER-2, or with A-431 cells as controls. IgE recognition of its target antigen leads to cross-linking of FcεRI, rat basophilic leukemia (RBL) cell degranulation, and thus, β-hexosaminidase release. Released enzyme was detected by 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MUG), and the resulting fluorescence measured at 465 nm (excitation wavelength 360 nm). For 100% release, RBL cells were lysed with Triton X-100, and relative experimental releases determined as follows:

$$\%\ \beta\text{–hexosaminidase release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Antibody-dependent cytotoxicity assay. The ADCC potential of the IgE antibodies induced by mimotope vaccination was assessed with a modified CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI). SK-BR-3 and A-431 cells were used as target cells; with A-431 cells as controls. The number of target cells was optimized to 2×10^5 cells/mL according to the instructions of the manufacturer. For the ADCC experiments, an effector to target (E/T) ratio of 100:1 was found to yield optimal specific lysis in a series of preliminary tests. RBL-2H3 cells were used as effector cells, after ascertaining that even at full ionomycin-induced degranulation, these cells do not release lactate dehydrogenase, the indicator of target cell lysis measured by a color reaction in this ADCC assay. Ten microliters of the third immune sera of the mimotope-immunized or the control mice were used for ADCC experiments. All assay procedures and readouts were done according to the instructions of Promega. The percentage of cytotoxicity was calculated after correcting for background absorbance values according to the following formula:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}}{\text{target maximum} - \text{target spontaneous}} \times 100$$

Maximal cytotoxicity caused by fully degranulated RBL cells against SK-BR-3 cells was set to 100% RBL-mediated lysis, and resulting cytotoxicities were adjusted accordingly. All titer determinations, β-hexosaminidase release, and ADCC assays were done in triplicate, and repeated for reproducibility.

Statistical analyses. Statistical analyses were done using two-tailed Student's t test, as immune responses in syngeneic mice were considered to be distributed normally. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly statistically significant.

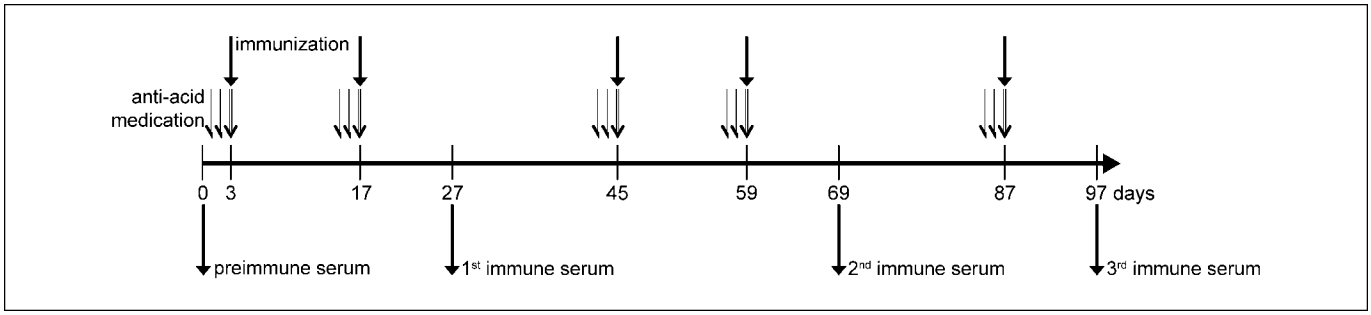

Figure 1. Oral immunization regimen. Timeline of antacid medication, antigen gavage, and blood sampling.

Results

Immune responses against the vaccine construct induced by oral mimotope vaccination. Mice were immunized with the mimotope C-QMWAPQWGPD-C or the carrier molecule KLH as shown in Fig. 1. As mimotopes on their own are too small to elicit an immune response, the immunogenic carrier KLH was used in designing the vaccine construct (26, 27). Groups were further designated as "QMW-KLH" and "KLH." To ensure hypoacidity during the gastric passage, mice received the proton pump inhibitor omeprazole once daily 2 days preceding each immunization, and twice on the day of immunization. Additionally, the antigens were dispersed in the protective film-building ulcer medication sucralfate. This combined regimen has been shown to effectively elevate gastric pH,[5] and lead to the formation of IgG and, importantly, IgE antibodies against food proteins fed under these conditions (23, 24). In contrast, proper digestion of dietary proteins favors immune tolerance or ignorance (28).

IgG and IgE titers against the carrier and trastuzumab mimotope were determined by DotBlot analysis. IgG titers reached a plateau after two courses of two immunizations, as no booster effect was seen between the second and third immune serum (see Fig. 1; Table 1). IgE was only first observed after these four immunizations, and could be boostered by an additional immunization (Table 1). A representative example of a DotBlot titer determination can be seen for the third immune sera in Fig. 2. Interestingly, both IgG and IgE responses were stronger against the mimotope than the carrier (in the case of IgE, there was no response against the carrier detectable at all, see Table 1).

IgE formation against the original antigen, HER-2. As shown in our previous work, a crucial point in mimotope immunizations is the induction of antibodies not only recognizing the applied mimotope, but also the original antigen that is mimicked. For anti-HER-2 IgG antibodies, we could show this cross-reactivity in Western blots (22). For IgE antibodies induced by the oral vaccination regimen, we employed another experimental setup. We applied an assay commonly used in allergology, i.e., a release assay from RBL cells. These cells only carry the high-affinity receptor for IgE, FcεRI, and can thus be used to determine IgE levels in sera in which a high content of IgG antibodies against the same antigen would otherwise interfere in assay sensitivity. When specific IgE is bound to the high-affinity receptors, and brought in contact with cognate antigen, FcεRI cross-linking and RBL degranulation occurs. The released enzyme β-hexosaminidase can be detected by a fluorescence reaction, and corresponds with the specific IgE content of the applied sera.

To show that the induced antibodies recognize the mimicked original antigen, HER-2, we used the HER-2–overexpressing breast cancer cell line SK-BR-3 as the source of antigen in the RBL assay. The HER-2-negative squamous cell carcinoma cell line A-431 was used as a negative control. IgE from mice immunized with QMW-KLH consistently led to highly significant degranulation of RBL cells after incubation with SK-BR-3 cells, but not with A-431 cells. KLH-immunized mice showed no reactivity with either cell line (Fig. 3). This indicates that the induced antimimotope IgE antibodies indeed specifically recognize the tumor antigen HER-2. Additionally, a successful RBL release assay was the first hint of the functionality of the induced IgE antibodies, as they have been shown to be capable of cross-linking FcεRI molecules.

IgE-mediated cytotoxicity. To further investigate the biological effects of the induced anti-HER-2 IgE antibodies, we adapted a classic nonradioactive cytotoxicity assay. RBL cells were used as effector cells, and sensitized with IgE from individual third immune sera from the QMW-KLH- and KLH-immunized mice. In this RBL ADCC assay, the mimotope-induced IgE antibodies exhibited RBL-mediated lysis in the range of 20% to 100% of SK-BR-3 cells (Fig. 4). RBL-ADCC ranging from 0% to 55% was seen with antibodies from the KLH-immunized control group. Although these ranges are broad and overlapping, the difference was found to be statistically significant ($P \leq 0.01$). When antimimotope antibodies were tested on A431 control cells, no specific cytotoxicity was observed (data not shown).

Discussion

The physiologic role of IgE antibodies is still not fully understood. Nevertheless, evidence is accumulating that these antibodies could be involved in the prevention of malignant disease

Table 1. Antibody titers of mimotope-immunized mice against the mimotope and the carrier protein

	IgG		IgE	
	QMW	KLH	QMW	KLH
Preimmune serum	—	—	—	—
First immune serum	1:1,000	1:1,000	—	—
Second immune serum	1:10,000	1:5,000	1:100	—
Third immune serum	1:10,000	1:5,000	1:500	—

[5] E. Untersmayer, unpublished data.

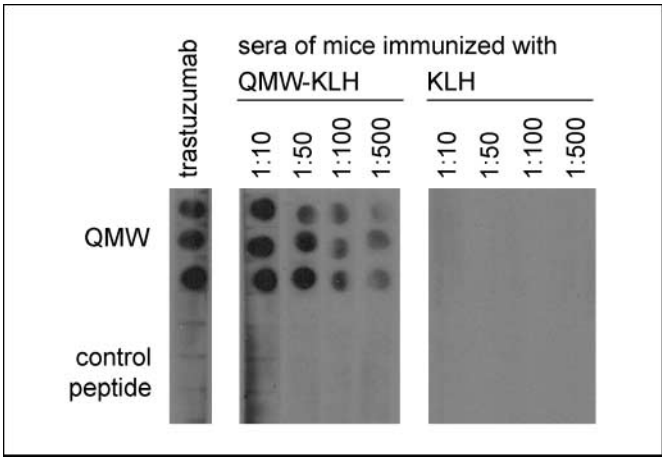

Figure 2. Oral immunization under acid suppression with the mimotope conjugate elicits IgE antibodies recognizing the immunogen. Mice were immunized with C-QMWAPQWGPD-C–KLH (*QMW-KLH*) or KLH alone. IgE titer determination by DotBlot against the peptide C-QMWAPQWGPD-C, and against a control peptide. Serum dilutions are shown.

(14–16). IgE is present in only low concentrations in the serum of normal individuals (~30 ng/mL; ref. 18), but is highly increased in two situations: the case of parasite infestation and in allergic disease. For both conditions, there is evidence of a negative association with cancer: Experimental data from mice show that animals infested with nematodes were resistant to syngeneic mammary adenocarcinoma (29), or showed decreased incidence of spontaneous mammary tumors (30). Huge epidemiologic studies and metaanalyses have been conducted on the topic of atopic disease and cancer risk, and a significant, if small, negative association has been found (14–16). The latest bit of evidence for the inverse association between allergy and malignant disease stems from the first clinical trials with the anti-IgE monoclonal antibody, omalizumab. Omalizumab is a human/murine chimeric monoclonal IgG antibody that prevents IgE from attaching to effector cells, thereby blunting IgE-mediated inflammatory responses. It is known to result in a marked reduction in serum levels of free IgE and down-regulation of IgE receptors on circulating basophils. When IgE was inhibited with this anti-IgE antibody, 1 in 200 asthmatic patients developed breast, skin, prostate or parotid gland malignancies during the median observation period of 1 year, whereas in the control group, the incidence was 1:500.[6]

The picture becomes clearer when one focuses on IgE specific for cancer cell antigens. There are early observations of "natural" IgE-mediated mechanisms in cancer patients. In 1977, Rosenbaum and Dwyer pointed out that evidence was published prior to the recognition of IgE that suggested that IgE may recognize tumor-specific antigens (13). A study in cancer patients in 1958 reported that eight tumor patients (five breast, two Hodgkin's, one lymphoma) showed wheal and flare skin reactions upon intradermal injections of lysates of their own tumor. This apparently was an immediate hypersensitivity response typically mediated by IgE. Moreover, this reaction could be transferred to a healthy volunteer in a classic Prausnitz-Küstner reaction (31). In 1972, Bartholomaeus and Keast observed that mice could produce IgE specific for a tumor, in this case, B16 melanoma (32).

Four groups have thus far targeted IgE to tumor-specific antigens, i.e., the mouse mammary tumor virus (17), a surface antigen of human colorectal carcinoma (18), the ovarian cancer antigen MOv18 (19, 21), and—via biotin-avidin bridging—to a lymphoma and an adenocarcinoma antigen (20). All found that IgE antibodies directly targeted to tumor antigens—in contrast with overall elevated IgE levels—cause a marked effect on tumor development and growth. For example, monoclonal IgE raised against murine mammary tumor virus were able to protect syngeneic mice from a lethal dose of mammary carcinoma cells (17), and as little as 1 μg of colon carcinoma–specific IgE per mouse was sufficient to inhibit COLO 205 tumor growth (18). However, all these strategies used passive applications of IgE. A vaccination regimen aimed at active induction of tumor-specific IgE seemed to be a promising approach.

Recently, we have developed an active immunization regimen for inducing tumor-inhibitory antibodies against HER-2, which is a member of the epidermal growth factor receptor (also known as the ErbB) family. This receptor is overexpressed in ~30% of breast cancer patients and confers a detrimental prognosis in the course of early as well as advanced breast cancer (33). The monoclonal antibody trastuzumab (Herceptin) targets this receptor, and

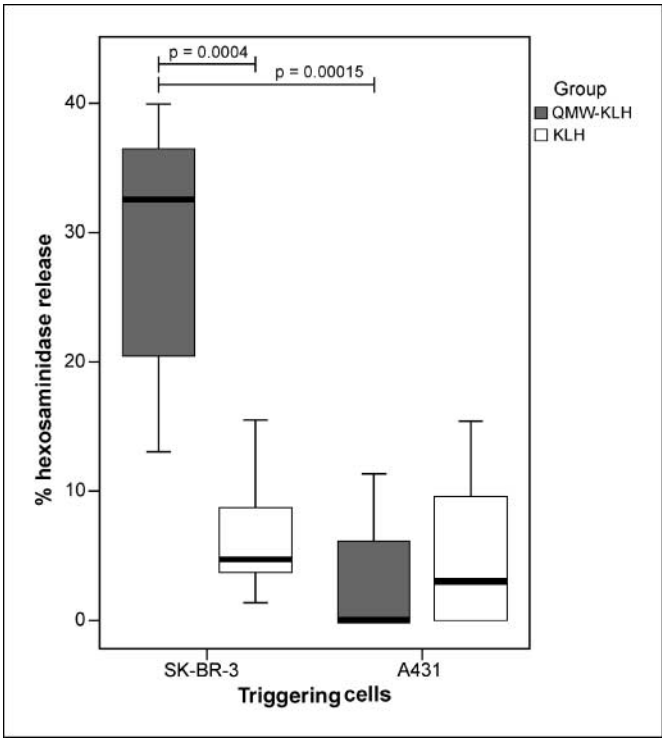

Figure 3. Mimotope-induced IgE antibodies recognize the original antigen, HER-2, expressed on SK-BR-3 breast cancer cells. In a β-hexosaminidase release assay with RBL-2H3 cells, IgE from mice immunized with C-QMWAPQWGPD-C–KLH (*QMW-KLH*) led to significant degranulation of RBL cells after incubation with HER-2–overexpressing SK-BR-3 cells, but not with the HER-2-negative control cell line, A-431. KLH-immunized mice show no reactivity with either cell line. *Box plots,* median, 25th, and 75th percentiles of β-hexosaminidase release mediated by sera of individual mice in each group ($n = 7$). This release correlates with the anti-HER-2 IgE levels of the respective groups. Both the difference of the mimotope-immunized group in SK-BR-3/A-431 recognition, as well as the difference between mimotope and control group in SK-BR-3 recognition, were found to be highly statistically significant ($P < 0.01$) by two-tailed Student's t test.

[6] Food and Drug Administration Briefing Document on Safety BLA STN 103976/0. http://www.fda.gov/ohrms/dockets/ac/03/briefing/3952B1_02_FDA-Xolair-Safety.pdf.

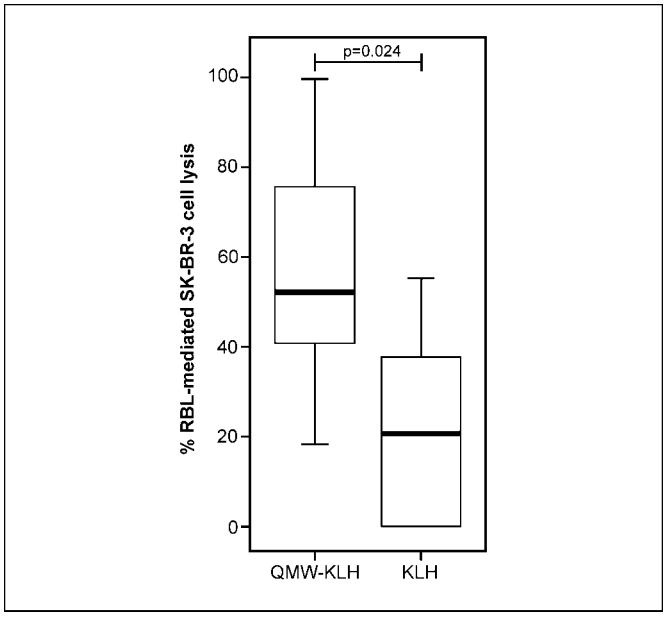

Figure 4. HER-2–specific IgE antibodies lead to SK-BR-3 breast cancer cell lysis via ADCC mechanisms. In a nonradioactive cytotoxicity assay, IgE-loaded RBL cells were used as effector cells. Mimotope-induced IgE antibodies targeting HER-2 mediated significant ADCC lysis of SK-BR-3 cells, when compared with control sera from KLH-immunized mice. *Box plots,* median, 25th, and 75th percentiles of relative target cell lysis (n = 7 per group). The difference between verum and control group was found to be statistically significant ($P < 0.05$) by two-tailed Student's t test.

beneficially influences the progression of early and advanced HER-2 overexpressing tumors. Consequently, we used trastuzumab to generate peptide mimics (so-called mimotopes) of its binding site on HER-2. These mimotopes induced trastuzumab-like IgG antibodies in mice upon parenteral (i.p.) immunization. Importantly, these actively induced antibodies showed similar biological antitumor features as the original antibody itself (22).

A second experience in our group stems from our research on food allergy mechanisms. We found that food proteins can effectively lead to IgE formation and sensitization when they persist the gastric passage undegraded (28). Gastric digestion is impaired in conditions of hypoacidity as, e.g., under antiulcer treatment. Consequently, an oral immunization regimen was developed that leads to the induction of a Th2 bias, i.e., high levels of food protein–specific IgG and IgE antibodies, eosinophilia, and hypersensitivity of skin and mucosa, when antigens are fed under concomitant gastric acid suppression (23, 24, 34).

In the present study, we combined our two fields of experience, and could show that feedings of trastuzumab mimotopes under antiacidic conditions indeed induced HER-2–specific IgE in BALB/c mice. These antibodies proved to be functional in an allergologic mediator release assay, where mimotope-induced IgE was found to react highly-specifically with HER-2–overexpressing breast cancer cells. To the best of our knowledge, this is the first active induction of tumor-specific IgE *in vivo*.

IgE is known to be capable of mediating ADCC of parasitic larvae in combination with effector cells bearing FcεRI. Indeed, these mechanisms play a major role in immune defense against parasites (35). Thus, IgE specifically targeted to tumor antigens could function in a similar way: by cross-linking FcεRI it would activate potent effector cells. Already in the first study on tumor-specific IgE, it was hypothesized that IgE-mediated cytotoxic mechanisms may play an immunologically specific antitumor surveillance role (17). Gould et al. also thought that IgE could mediate anticancer ADCC, and therefore created chimeric IgE and IgG_1 antibodies recognizing an ovarian cancer antigen to directly compare between the two subclasses. Indeed, in their *in vivo* model, only IgE, and not IgG_1 caused sustained inhibition of tumor growth until the end of the experiments (day 48). The inhibitory effect of tumor-specific IgE was substantial (83%) and highly significant (19). In a more recent publication, they were able to show that human monocytes were active in IgE-dependent ADCC *in vivo*. Tumor cell killing was proportional to the expression of FcεRI on monocytes, and they observed phagocytosis of tumor cells by the monocytes *in vitro*. Testing basophils, they observed targeted exocytosis of the granules at the site of contact with cancer cells, and overall, an accumulation of basophils at the site of tumors (21).

We therefore did an assay evaluating the ADCC-mediating potential of the induced anti-HER-2 IgE antibodies towards breast cancer cells. As effectors, we used the rat basophilic leukemia cell line RBL-2H3, which expresses the rodent FcεRI as its only antibody receptor. Thus, we were sure that no other antibodies contained in the immune sera could interfere in this assay. These cells were incubated with individual serum samples of mimotope and KLH immunized mice. Indeed, specific IgE-mediated lysis of target cells could be observed. The degree of IgE-ADCC mediated by the sera correlated with the level of HER-2-specific IgE detected by the β-hexosaminidase assay.

Generally, IgE may have two major advantages over other isotypes in the therapy of cancer. One aspect may be the prolonged duration of antitumor effects. Whereas the serum half-life of unbound IgE in humans is only ~2 days, the half-life of IgE in skin is in the order of several months (36). Thus, IgE-mediated effector functions at tissue tumor sites could be expected to last for a long time. The extended lifetime of receptor-bound IgE may allow cells to locate and kill tumor cells in solid tissues more effectively than IgG.

The second aspect is the high affinity of the interaction between the Fc portion of IgE and FcεRI, which may allow effective arming of potential effector cells that would then be able to extravasate and penetrate tissue without losing surface-bound antibody (18). Indeed, it is well known that there is an infiltration of developing tumors by various FcεRI-bearing immune cells. More importantly, on-site production of IgE by plasma cells could contribute to beneficial effects. Indeed, a high number of IgE-positive cells was found in most squamous cell carcinomas of the head and neck when compared with normal mucosa (37).

In general, the special role of IgE is to act as a very potent inducer of mediator release and cytokine production from eosinophils, basophils, macrophages, and mast cells. Among the released substances are histamine, heparin, the enzymes tryptase and chymase, as well as the proinflammatory cytokines granulocyte macrophage colony-stimulating factor, interleukin 4, interleukin 5, and tumor necrosis factor α (38, 39). Moreover, several lines of evidence indicate a crucial role for eosinophils and macrophages in tumor eradication through a mechanism involving the production of reactive oxygen metabolites and nitric oxide (20). But in humans, it has to be considered that the high-affinity receptor FcεRI is also expressed on antigen-presenting cells as Langerhans cells and monocytes (40). IgE is capable of mediating antigen capture, processing, and presentation by professional antigen-presenting cells, facts known from allergic patients. Thus, IgE-driven antitumor activity is not restricted to the activation of innate

immunity effector mechanisms, but could also initiate efficient antigen presentation and thus priming of a T cell–mediated adaptive immune response (41). It is likely that IgE antibodies trigger an inflammatory reaction at the tumor site by recruitment and activation of FcεR-bearing effector cells, which in turn, may favor tumor cell destruction, an essential requirement for efficient priming of CD4- and CD8-dependent immune responses (42, 43).

Taken together, there is growing evidence that IgE might have a role in cancer surveillance and suppression. The negative association between cancer incidence and conditions with elevated IgE levels could indicate that atopic individuals possibly have some antitumor protection due to a tendency to form IgE antibodies, and among them, the beneficial tumor-specific ones as well. The slightly increased cancer risk seen in patients exposed to the anti-IgE antibody omalizumab[6] might thus be explained by both a reduction of possible tumor-specific IgE antibodies, but even more so, by its reduction of FcεR-bearing effector cells.

We conclude that IgE specifically targeted to tumor antigens elicits effective antitumor immune responses and that it is feasible to induce tumor antigen–specific IgE antibodies by active immunization.

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CHAPTER V

Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/*neu*-positive tumour cells

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ORIGINAL ARTICLE

Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER2/*neu*-positive tumour cells

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Abstract Trastuzumab (Herceptin®), a humanized IgG1 antibody raised against the human epidermal growth factor receptor 2 (HER2/*neu*), is the main antibody in clinical use against breast cancer. Pre-clinical evidence and clinical studies indicate that trastuzumab employs several anti-tumour mechanisms that most likely contribute to enhanced survival of patients with HER2/*neu*-positive breast carcinomas. New strategies are aimed at improving antibody-based therapeutics like trastuzumab, e.g. by enhancing antibody-mediated effector function mechanisms. Based on our previous findings that a chimaeric ovarian tumour antigen-specific IgE antibody showed greater efficacy in tumour cell killing, compared to the corresponding IgG1 antibody, we have produced an IgE homologue of trastuzumab. Trastuzumab IgE was engineered with the same light- and heavy-chain variable-regions as trastuzumab, but with an epsilon in place of the gamma-1 heavy-chain constant region. We describe the physical characterisation and ligand binding properties of the trastuzumab IgE and elucidate its potential anti-tumour activities in functional assays. Both trastuzumab and trastuzumab IgE can activate monocytic cells to kill tumour cells, but they operate by different mechanisms: trastuzumab functions in antibody-dependent cell-mediated phagocytosis (ADCP), whereas trastuzumab IgE functions in antibody-dependent cell-mediated cytotoxicity (ADCC). Trastuzumab IgE, incubated with mast cells

Panagiotis Karagiannis and Josef Singer contributed equally to this work.

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and HER2/*neu*-expressing tumour cells, triggers mast cell degranulation, recruiting against cancer cells a potent immune response, characteristic of allergic reactions. Finally, in viability assays both antibodies mediate comparable levels of tumour cell growth arrest. These functional characteristics of trastuzumab IgE, some distinct from those of trastuzumab, indicate its potential to complement or improve upon the existing clinical benefits of trastuzumab.

Keywords HER2/*neu* · Trastuzumab · IgE · Monocytes · Mast cells · Tumour immunity

Abbreviations

HER2/*neu*	Human epidermal growth factor receptor 2
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cell-mediated phagocytosis
FBP	Folate binding protein
sFcεRIα	Soluble FcεRI α-chain
ECD^{HER2}	HER2 protein extracellular domain
CM	Complete medium
PI	Propidium iodide
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
NIP	4-Hydroxy-3-nitro-phenacetyl
PI3K	Phosphoinositide 3-kinase
TGF-a	Tumour growth factor α
VEGF	Vascular endothelial growth factor
TNF-α	Tumour necrosis factor-α
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)
PMS	Phenazine methosulfate

Introduction

HER2/*neu* (c-erb-B2) is a 185 kDa protein that belongs to the human epidermal growth factor receptor family. Its functions include engendering cell signalling and regulating cell growth, proliferation, differentiation and motility [1]. Approximately 30% of breast carcinomas as well as other cancers, such as those of the ovary, endometrium, bladder, prostate and lung, over-express HER2/*neu*, whilst its expression in normal tissues is low [2]. Its expression in breast cancer is thought to play a vital role in the pathogenesis of breast tumours and is linked to poor clinical outcomes [3]. This antigen is now a validated target for cancer therapeutics.

Trastuzumab (Herceptin®) is an IgG1 antibody raised against the extracellular domain (ECD) of HER2/*neu* and is the main antibody in clinical use for the treatment of HER2-positive breast cancers [4–6]. Trastuzumab was approved by the FDA in 1998 for the treatment of metastatic HER2/*neu* over-expressing breast cancer and is now also used as adjuvant therapy for early breast cancers. The success of trastuzumab in breast cancer therapy has renewed interest in antibody therapies and provoked further research into the development of therapeutic antibodies. However, only a subset of patients treated with trastuzumab show significant responses and thus there is scope for additional modalities designed to improve clinical outcomes [7].

Trastuzumab is thought to exert anti-tumour effects by a number of mechanisms. The best-defined mechanism is the blocking of the hetero-dimerization of HER2/*neu* receptors with other HER family members (HER1, HER3) on the surface of breast cancer cells thereby switching off vital tumour cell growth signals [8, 9]. Trastuzumab inhibits metalloproteinase activity and interferes with signalling via phosphoinositide 3-kinase (PI3 K) pathways, promoting apoptosis and cell cycle arrest during the G1 phase. Another mechanism is thought to be blocking angiogenesis by inducing expression of anti-angiogenic factors such as thrombospondin-1 and suppression of pro-angiogenic factors such as TGF-α, VEGF, angiopoietin-1, and plasminogen-activator inhibitor-1 [10]. Clinical and pre-clinical studies suggest that trastuzumab may also enlist immune effector cells to attack and kill tumour cells by cytotoxicity (ADCC) and phagocytosis (ADCP), and by augmenting chemotherapy-induced cytotoxicity [11–14]. Studies are now focusing on strategies aimed at improving the significant but circumscribed success of trastuzumab. These include optimising antigen specificity or affinity and enhancing antibody-mediated effector cell functions targeted against tumour cells.

Although there are five antibody classes in man, each with distinctive functions in the immune system, trastuzumab, but essentially all monoclonal antibodies approved for clinical use are IgG1 s. Antibodies of the IgG class function most effectively in the circulation [15]. There are many reasons why IgE antibodies might be more effective against tumours that develop in tissues and are therefore inaccessible to IgGs [16]. The concentration of IgE in the serum of normal individuals is minute (<150 ng/mL, 1/10,000 the concentration of IgG) because IgE is sequestered in solid tissues, where it is bound with high-affinity to receptors on its effector and antigen-presenting cells [17]. The affinity of IgE for FcεRI (Ka $\sim 10^{11}$ M^{-1}) is 10^2–10^5 times higher than those of IgGs for their receptors, making IgE the only antibody strongly retained by effector cells in the absence of antigen [17, 18]. The half-life of IgE in tissues (measured in the skin ~2 weeks) is longer than that of IgG (2–3 days) [18, 19]. IgE saturates FcεRI at nM concentrations, but only 10% of the receptors need be occupied by IgE and antigen for full mast cell activation and effector cell

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recruitment to the site of antigen challenge in tissues [17, 20]. IgE antibodies on the surface of tissue mast cells are cross-linked by antigens to induce the release of histamines, leukotrienes, proteases, and, importantly, Th2 cell-type cytokines (IL-3, IL-4, IL-5, IL-6, IL-9, IL-13, TNF-α) at the site of antigen challenge. This results in activation of the resident immune effector cells, but also elicits further recruitment and persistence of an inflammatory cell infiltrate, comprising Th2 cells, monocytes, eosinophils and basophils, from the circulation, which enhances and maintains the local immune response [17]. IgE antibodies directed against a tumour-associated antigen would therefore trigger an immediate local effector cell response against tumour cells and stimulate a cascade of inflammation targeted to the tumour cells in situ. These activities could possibly be highly effective in immune rejection of tumours embedded in solid tissues.

Several studies support the ideas IgE antibodies may be highly effective tools in cancer therapy [21–28]. We have previously shown that an antibody of the IgE class is superior to the corresponding IgG1 antibody against folate binding protein (FBP) in prolonging survival of mice in two xenograft models of ovarian cancer [29–32]. Ours and other studies [23, 27, 33–35] have contributed to the suggestion that the antibody class may influence the nature as well as the potency of the immune responses elicited against tumour cells.

In order to examine the mechanisms by which an IgE version of trastuzumab may act in tumour cell killing, we have engineered a humanised trastuzumab IgE. Here, we report the physical characterisation and functional properties of the engineered trastuzumab IgE, and show that these properties are distinct from those of trastuzumab (IgG1). Our data suggests that trastuzumab IgE may potentiate tumour killing by mechanisms and pathways that might be highly effective in cancer therapy.

Materials and methods

Antibodies and reagents

Chimaeric mAbs MOv18 IgE and MOv18 IgG (IgG1 isotype) against the human folate binding protein (FBP), NIP IgE specific for the hapten 4-hydroxy-3-nitro-phenacetyl (NIP) and the recombinant IgE receptor FcεRI alpha (sFcεRIα) were prepared as before [29, 36, 37]. ECD^{HER2}, the soluble human HER2 protein comprising the HER2/*neu* extracellular domain (ECD) (90 kDa) was prepared as previously described [38]. Trastuzumab (Herceptin®) was from Genentech (San Francisco, CA, USA), goat anti-human IgE-FITC was from VECTOR Laboratories Ltd. (Peterborough, UK) and anti-CD89-PE and anti-CD33-APC mAbs were from BD Biosciences (Oxford, UK). Antibodies to Fcε and Fcγ receptors, human IgG isotype-matched control and goat anti-mouse-Ig-FITC Abs were from Dako (Glostrup, Denmark). PI, CFSE, and tissue culture reagents were from Invitrogen (Paisley, UK).

Generation of trastuzumab IgE antibody

The cDNA derived from the protein sequences of the heavy and light chains of the trastuzumab variable regions was synthesised (Gene Art AG, Regensburg, Germany) based on the published protein sequence of trastuzumab (source: http://www.pdb.org; 1n8z) [39]. This cDNA was then cloned into two vectors based on a pTT vector backbone, one containing the epsilon heavy chain of IgE (humighae2, accession no: L00022; Kenten et al. 1982), the other containing the human kappa light chain constant region cDNA (IGKC, accession no: BC110394) [40, 41] (Fig. 1). For full amino acid sequences for trastuzumab IgE see Supplementary Table I (Supplementary Data). For transfection into compatible HEK293 cells, vector DNA was produced using the HiSpeed Plasmid Maxi Kit® (Qiagen®), according to the manufacturer's instructions. HEK293 cells were harvested and seeded at 4×10^5 cells/mL and allowed to adhere before being transfected with 1 μg of DNA with the aid of 2 μg of PEI (Polyethenylenimine, MW: 25 kDa; Polysciences Inc., Warrington, PA, USA) per 4×10^7 cells [40]. Supernatants were harvested 2–4 weeks later and antibodies were purified by affinity chromatography as previously described [29]. Antibody purity was confirmed by HPLC analysis.

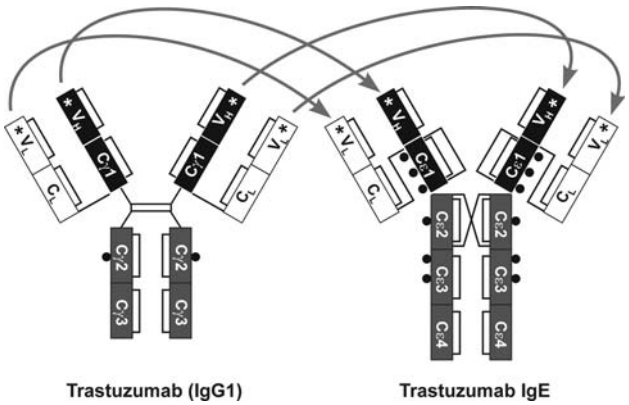

Fig. 1 Schematic representation of the design of trastuzumab IgE antibody. To engineer trastuzumab IgE, the variable heavy and light chains of trastuzumab (IgG1, *left*; regions indicated with *stars*) were inserted into the epsilon heavy chain regions of IgE and the epsilon heavy chain was combined with the kappa light chain to produce the corresponding trastuzumab IgE antibody (*right*). The resulting engineered IgE molecule should recognise the HER2/*neu* antigen and IgE receptors (see Supplementary Table I, Supplementary Data for full sequence). Glycosylation sites are depicted by *black circles*

Kinetic assays of antibody binding to HER2 and FcεRI

Kinetic studies were performed using surface plasmon resonance (SPR) analysis to examine the specificities and binding affinities of trastuzumab (IgG1) and trastuzumab IgE antibodies to the soluble ECD^{HER2} protein and to the soluble high-affinity IgE receptor alpha subunit (sFcεRIα), each immobilised on the biosensor surface. Kinetics of trastuzumab IgE binding to immobilised FcεRIα on the biosensor surface were compared to the well-characterised chimaeric antibody NIP IgE. All experiments were performed at 24°C on a Biacore 3000 instrument (Biacore Int. SA, Switzerland). Methods and kinetic analysis have been described previously [36, 42, 43]. In these experiments, antibodies were tested at a concentration range of 125–7.8 nM, coupling density was typically restricted to 200RU, flow rate 20 μL/min, and exposure time to analyte 360 s.

Cell culture

The human monocytic cell line U937 [44] (kindly provided by Prof. J.-P. Kinet, Harvard University, Boston, MA, USA) was grown in RPMI 1640 medium, 10% FCS, 2 mM l-glutamine, penicillin (5,000 U/mL) and streptomycin (100 μg/mL). The murine colon adenocarcinoma cell lines CT26 [45, 46] and CT26-HER2/neu^{Her2+} transfected with the HER2/*neu* antigen [47] were grown in Iscove's medium (IMDM), 5% FCS, 2 mM l-glutamine, penicillin (5,000 U/mL) and streptomycin (100 μg/mL). The human breast adenocarcinoma cell line SKBR3 (ATCC, No. HTB-30), that naturally expresses the HER2/*neu* antigen, was grown in DMEM, 10% FCS, 2 mM l-glutamine, penicillin (5,000 U/mL) and streptomycin (100 μg/mL). The rat basophilic leukaemia mast cell line RBL-SX38 [48] (Prof. J.-P. Kinet, Harvard University, Boston, MA, USA) expresses the human form of the FcεRI receptor and was maintained in MEM supplemented with 10% FCS, 250 μg/mL Geneticin, 2 mM l-glutamine, penicillin (5,000 U/mL) and streptomycin (100 μg/mL). All cells were maintained at 37°C in 5% CO_2.

Flow cytometric assessments of antibody binding to receptors on cells

For flow cytometric assessments of antibody binding to the tumour-associated antigens FBP and HER2/*neu* and to Fcε (IgE) and Fcγ (IgG) receptors on receptor-expressing cells, cells were incubated with 0.5 μg/mL mAbs for 30 min at 4°C, followed by two washes in FACS buffer (PBS, 5% normal goat serum). Cells were then given anti-human IgE-FITC or anti-human IgG-FITC (10 μg/mL) for 30 min at 4°C, washed in FACS buffer and fixed in 1% paraformaldehyde-FACS buffer prior to acquisition and analysis on a dual laser FACSCalibur™ (BD Biosciences). For quantitative assessments of Fcε and Fcγ cell surface receptors, 2×10^5 U937 cells/sample were stained with mouse mAbs by indirect immunofluorescence using the QIFIKIT® (Dako). U937 cells, setup beads and calibration beads were given goat anti-mouse IgG-FITC, followed by two washes in FACS buffer and analysis by flow cytometry on a FACSAria II flow system (BD Biosciences). The numbers of receptors per cell were calculated against fluorescent calibrating bead standards using linear regression.

Flow cytometric ADCC/ADCP assay

Treatment of tumour cells

We employed our previously described novel three-colour flow cytometric assay to simultaneously measure tumour cell cytotoxicity (ADCC) and phagocytosis (ADCP) of HER2/*neu*-positive tumour cells by human effector cells [30, 31, 49]. CT26-HER2/*neu* or SKBR3 cells were stained 24 h prior to assays with 7.5 μM CFSE in PBS for 10 min at 37°C, washed in RPMI 1640 medium, 10% FCS, 2 mM L-glutamine, and returned to normal culture conditions. The following day, CFSE-labelled tumour cells were washed, mixed with unstained effector cells at E:T ratio of 2:1 with or without antibodies, followed by incubation for 2.5 h at 37°C. Antibodies were tested at concentrations of 0.05, 0.5 and 5 μg/mL.

Three-colour flow cytometric assay setup

CFSE-labelled tumour cells were detected in FL1 (530/30 nm band pass filter), PE-labelled monocytic effector cells in FL2 (582/42 nm band pass filter) and PI^+ dead cells in FL3 (670 nm LP band pass filter) channels, whilst control samples were set for compensation adjustments between fluorochromes. Two dual colour flow cytometric dot plots were generated to calculate ADCC and ADCP as previously described [30, 31, 49]. Briefly, one dot plot depicted CFSE + tumour cells and PI + cells, allowing quantitation of tumour targets killed externally by effector cells (ADCC, cytotoxicity) (CFSE +/PI + cells). The second dot plot depicted CFSE + tumour cells and CD89-PE + effector cells in order to quantitate total CFSE + tumour cells and the number of tumour cells present within PE + effector cells, depicting phagocytosis (ADCP) by effector cells (CFSE +/PE + cells) [49]. This dot plot would also indicate any non-specific uptake of CFSE fluorescence by PE + U937 effector cells.

Confocal imaging of cell contact and antibody-mediated tumour cell phagocytosis

U937 monocytes, which served as effector cells, were incubated on Lab-Tec II glass chamber slides (SLS Ltd,

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Manchester, UK) with CFSE-labelled CT26-HER2/*neu* tumour cells at an original E:T ratio of 2:1. Treatments were performed as above. Mixed cell cultures were incubated for 3 h with MOv18 IgG, MOv18 IgE, trastuzumab (IgG1) or trastuzumab IgE antibodies. At the end of the incubations, cells were then given anti-CD33-APC for 40 min at 4°C, to label monocytic cells. Cells were then washed, fixed in 4% paraformaldehyde-FACS buffer and mounted with fluorescence preserver (Dako). Fluorescence microscopy was performed on a Zeiss Axiovert 200 confocal microscope (63 × oil immersion objective). Acquisition and analysis was performed with UltraView software (PerkinElmer, Waltham, MA, USA).

In vitro degranulation assays

The ability of the engineered trastuzumab IgE to trigger degranulation was measured in vitro using the rat basophilic mast cell line RBL-SX38. This cell line expresses the human form of the FcεRI receptor as a $\alpha\beta\gamma_2$ tetramer, the form naturally expressed on the surface of human mast cells [48, 50, 51]. For degranulation experiments cells were plated at 2×10^4 per well in 100 µL in 96 well flat-bottomed tissue culture plates and incubated overnight at 37°C in a humidified CO_2 incubator. The following day, cells were sensitised with IgE diluted in culture medium at 100 ng/mL, incubated for two hours at 37°C and washed twice with HBSS, 1% BSA (wash buffer). Cell degranulation was triggered for 30 min either with 100 µL of anti-human IgE polyclonal rabbit mAb (Dako) (final concentration: 100 ng/mL), or HER2/*neu*-expressing CT26 cells added at different concentrations (1×10^3 to 5×10^5 per well) in wash buffer at 37ºC. Degranulation was terminated by placing the cells on ice and the supernatants removed for quantification of mediator release. Control supernatants were either from individual or mixed cell populations alone treated with no antigen for background release, or wash buffer plus 0.1% Triton-X-100 (Tx) for total release. Degranulation was measured by quantification of β-hexosaminidase release, assayed using a fluorogenic substrate (4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide) prepared according to a standard protocol [51, 52]. Supernatants were incubated in black 96 well plates with an equal volume of substrate at 37°C for 2 h and quenched with 0.5 M Tris. Fluorescence was measured using a Thermo Fluoskan II fitted with a 350 nm excitation and a 450 nm emission filters. All measurements were made in triplicate for each concentration and release was expressed as a percentage of total content determined by treatment with 0.1% Triton-X-100 solution in HBSS, 1% BSA. Background release, subtracted from all values, was always <10% of total release.

Cell viability assay

Tumour cell viability was analysed by the MTS assay (tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) using phenazine methosulfate (PMS) as a reducing agent (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit, Promega, Southampton, UK). Cells were seeded in 96-well plates at 4×10^4 cells per well and allowed to adhere overnight under standard culture conditions prior to assays. Cells were exposed to 0.5 µg/mL trastuzumab, trastuzumab IgE, MOv18 IgG or MOv18 IgE antibodies over a period of 4, 24, and 48 h. Control groups received media alone, or 0.9% v/v Triton-X-100 for 30 min prior to addition of MTS. Following treatments, MTS/PMS solution prepared according to the manufacturer's instructions were added at 20 µL per well and cells were incubated for a further 1 h prior to recording absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

Data handling and statistical analysis

In surface plasmon resonance assays, mean values ±SD were calculated from three measurements. Flow cytometry experiments of receptor binding and blocking were repeated at least three times. In vitro ADCC/ADCP assays were performed in triplicate and data are shown as mean ADCC ± SD and ADCP ± SD of a number (n) of independent experiments (see Supplementary Table II in Supplementary Data). Statistical analyses of in vitro ADCC/ADCP assays and microscopic measurements of effector-tumour cell interactions were performed by means of the unpaired two-tailed Student's t test, and significance was accepted at $P < 0.05$.

Results

Binding of trastuzumab IgE to antigen and FcεRI

We have compared the kinetics of binding of the engineered trastuzumab IgE and trastuzumab (IgG1) to HER2/*neu* ECD^{HER2} immobilised on a biosensor surface by surface plasmon resonance (Fig. 2a; Table 1). The two antibodies exhibited similar rates of association and dissociation from their complexes with ECD^{HER2} (k_a, k_d, mean ± SD, Table 1). These data demonstrate that trastuzumab IgE exhibits the expected interaction with ECD^{HER2}, and the calculated affinity values of both trastuzumab IgE and IgG1 (Ka of 10^{10}) are similar to those

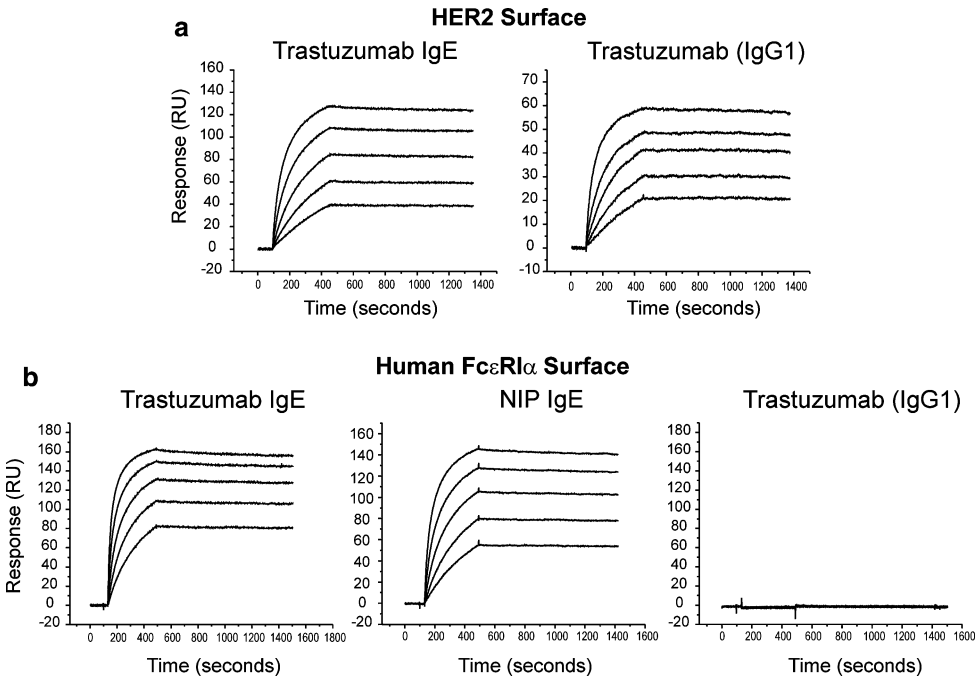

Fig. 2 Comparative SPR analysis of trastuzumab IgE and trastuzumab (IgG1) kinetics of binding to immobilised HER2 receptor ECD^{HER2} (**a**) and to immobilised FcεRIα (**b**). Data were recorded using a Biacore 3000 (flow rate 20 μL/min). Antibodies were tested at concentrations ranging from 125 to 7.8 nM. All values derived from the fitting procedures are given in Table 1

Table 1 Calculated kinetic values of trastuzumab IgE binding to HER2 and FcεRI

Constant	ECD^{HER2} surface		sFcεRIα surface	
	Trastuzumab IgE	Trastuzumab (IgG1)	Trastuzumab IgE	NIP IgE
k_a (M^{-1} s^{-1})	$(3.5 \pm 1.4) \times 10^5$	$(7.3 \pm 3.5) \times 10^5$	$(4.8 \pm 2.3) \times 10^5$	$(2.3 \pm 1.1) \times 10^5$
k_d (s^{-1})	$(2.4 \pm 0.3) \times 10^{-5}$	$(1.8 \pm 0.9) \times 10^{-5}$	$(2.3 \pm 0.5) \times 10^{-5}$	$(2.6 \pm 0.5) \times 10^{-5}$
Ka (M^{-1})	1.5×10^{10}	4.2×10^{10}	2.1×10^{10}	1.0×10^{10}

Kinetic parameters and affinity constants derived from the SPR analysis of NIP IgE and trastuzumab IgE binding to immobilised sFcεRIα and trastuzumab (IgG1) and trastuzumab IgE binding to immobilised ECD^{HER2}. Both IgEs and trastuzumab were analysed using a 1:1 model of association from which association and dissociation constants were derived for each component (shown ± SD for at least five determinations in the concentration range 125–7.8 nM)

previously reported for the IgG1 [6, 53]. Comparison of sensograms representing binding of trastuzumab IgE and the chimaeric NIP IgE to the immobilised soluble alpha-chain of FcεRI (sFcεRIα) demonstrates that both antibodies bind to the high-affinity IgE receptor with the expected kinetics and affinity (k_a, k_d, mean ± SD; Fig. 2b; Table 1). In particular, both antibodies demonstrate the documented slow dissociation rate that is characteristic of the complex of IgE with FcεRI (Fig. 2b) [36, 42, 43]. As expected, trastuzumab (IgG1) did not bind to the IgE receptor (Fig. 2b, right panel).

Trastuzumab interaction with receptors on monocytic effector and tumour cells

Flow cytometric assessments of trastuzumab and trastuzumab IgE interactions with HER2/*neu* and Ig (Fcγ and Fcε) receptors on the surface of cells served two purposes. The first was to confirm that the antibodies recognise their native receptors as presented on cell surfaces. The second was to explore the mechanisms employed by trastuzumab and trastuzumab IgE together with human monocytic cells to target and kill HER2/*neu* receptor-expressing tumour cells. For this, we analysed the interactions of these antibodies with U937 monocytic cells, which served as effector cells, and with the CT26 cells transfected to express the human HER2/*neu* receptor on the cell surface (CT26-HER2/*neu*) [46], used as target cells.

The expression of IgG receptors FcγRI, FcγRII and FcγRIII and of IgE receptors FcεRI and CD23 on U937 cells were also measured (Table 2). U937 monocytes express FcγRI (~12,000 molecules per cell) and FcγRII (~19,000 molecules per cell) but very low levels of FcγRIII (~700 molecules per cell). In agreement with our previously published data [30], we measured approximately 22,000 molecules of FcεRI are expressed per cell, whilst the expression of CD23 was low (~2,200 molecules/cell).

Consistent with the abundant expression of Fcγ receptors on the surface of U937 cells, trastuzumab bound to Fcγ receptors expressed on the surface of 99.8% of U937 mono-

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Table 2 Quantification of IgE and IgG Receptors on U937 Monocytes

Surface antigen	Number of molecules per cell (mean ± SD) ($n = 9$)
CD23	$2.2 \times 10^3 \pm 1.3 \times 10^3$
FcεRI	$21.8 \times 10^3 \pm 4.8 \times 10^3$
FcγRI	$12.1 \times 10^3 \pm 3.9 \times 10^3$
FcγRII	$19.0 \times 10^3 \pm 3.9 \times 10^3$
FcγRIII	$0.7 \times 10^3 \pm 0.4 \times 10^3$

cytic cells (Fig. 3a, upper left), as did the chimaeric antibody MOv18 IgG, specific for the ovarian tumour antigen FBP, used as positive control (Fig. 3a, upper right). Trastuzumab also bound to 79.8% of CT26-HER2/*neu* cells (Fig. 3a, bottom left), whilst only background binding of the MOv18 IgG was detected (5.2%) (Fig. 3a, bottom right). These data confirm the specificity of the antibody for the human HER2/*neu* antigen expressed on the surface of tumour cells as well as to the Fcγ receptors expressed by monocytic cells.

Trastuzumab-mediated killing of tumour cells

We employed our previously developed three-colour flow cytometric assay to simultaneously measure trastuzumab ADCC and ADCP of HER2/*neu*-expressing tumour cells [49] (Fig. 3b, c). The CT26-HER2/*neu* cells were used as tumour targets and human U937 monocytes were employed to provide effector cells. The green-fluorescent dye CFSE was used to stain live CT26-HER2/*neu* cells prior to incubation with U937 monocytes and, after the incubation, U937 cells were stained with anti-CD89-PE and dead cells with propidium iodide (PI). Two colour flow cytometric dot plots show that after 2.5 h in culture, U937 cells mixed with trastuzumab and CT26-HER2/*neu* mediated little ADCC above that seen with samples incubated with the MOv18 IgG (5.3 vs. 4.8%; Fig. 3b, top panels, top right boxes for double positive CFSE +/PI + cells). Two-colour flow cytometric dot plots also showed that incubation with trastuzumab induced appreciable tumour cell ADCP compared to control MOv18 IgG (30.6 vs. 4.9%; Fig. 3b, bottom panels; top right boxes for double positive CFSE +/PE + cells). Flow cytometric ADCC/ADCP assay measurements confirmed that trastuzumab at an optimal concentration of 0.5 μg/mL mediated significant levels of ADCP of CT26-HER2/*neu* tumour cells by monocytic cells. Levels of ADCP increased ~10% above those of the MOv18 IgG and no antibody controls (Fig. 3c and Supplementary Table II, Supplementary Data). Statistically significant levels of ADCC were not measured with any of the conditions tested here (Supplementary Table II, Supplementary Data). These data suggest that trastuzumab-IgG1 mediates ADCP, but not ADCC of tumour cells by monocytic cells.

Cell viability assays demonstrated that CT26-HER2/*neu* tumour cells were susceptible to the anti-proliferative effects of trastuzumab (IgG1). These effects were detected after 24 h (89 vs. 96.6% cell viability for trastuzumab and MOv18 IgG, respectively) and 48 h incubation (87.5 vs. 104.5% cell viability for trastuzumab and MOv18 IgG, respectively). No cell growth arrest was detected with trastuzumab after exposure to antibody for 4 h (104.6 and 100% viability for trastuzumab and MOv18 IgG, respectively (Supplementary Fig. 1, Supplementary Data). This confirmed that tumour cell death measured with the ADCC/ADCP assays after 3 h exposure to antibodies was not the result of receptor hetero-dimerisation blocking by trastuzumab alone. Thus, the rapid cell death detected by the ADCC/ADCP assay was most likely mediated by Fcγ receptors on U937 monocytes in combination with trastuzumab.

The tumour-targeting and phagocytic activities of trastuzumab measured in the ADCC/ADCP assays were confirmed by confocal microscopical imaging (Fig. 3d). CT26-HER2/*neu* cells were pre-labelled with CFSE (green), incubated with U937 cells at and E:T ratio of 2:1, combined with antibodies, incubated for 3 h on glass chamber slides, and U937 cells were labelled with anti-CD33-APC mAb (red). In samples incubated with trastuzumab, enhanced contact between CT26-HER2/*neu* tumour cells (green) and U937 monocytic cells (red) was evident, and in many instances two or more monocytic cells were observed in contact with a single tumour cell (Fig. 3d, left, white arrows). We also observed phagocytosis of tumour cells, clearly visible in the merged image of the green tumour cells inside the red U937 cells: most monocytic cells in contact with tumour cells appeared to contain tumour cell material (Fig. 3d, left; white arrows). In contrast to these observations, neither contact nor phagocytosis were observed in samples given MOv18 IgG examined after 3 h in the same culture conditions (Fig. 3d, right). To confirm the microscopic observations, the frequency of interactions between effector and target cells were measured (Table 3). Incubation with trastuzumab led to enhanced contact (24.5% of tumour cells) between tumour and U937 monocytes after 3 h compared to 7.9% contact observed in samples incubated with MOv18 IgG. Most of the U937 monocytes in contact with tumour cells contained tumour cell material (20.4% of tumour cells), suggesting tumour cell phagocytosis, rarely seen with MOv18 IgG (3.5%). Findings from microscopic observations and measurements of cell-cell interactions are in agreement with our ADCC/ADCP assays suggesting that trastuzumab mediated tumour cell killing by phagocytosis.

Trastuzumab IgE interaction with HER2/*neu* and IgE receptors on monocytic effector cells and tumour cells

We analysed the interactions of trastuzumab IgE with its Fcε receptors on U937 monocytic cells and with human

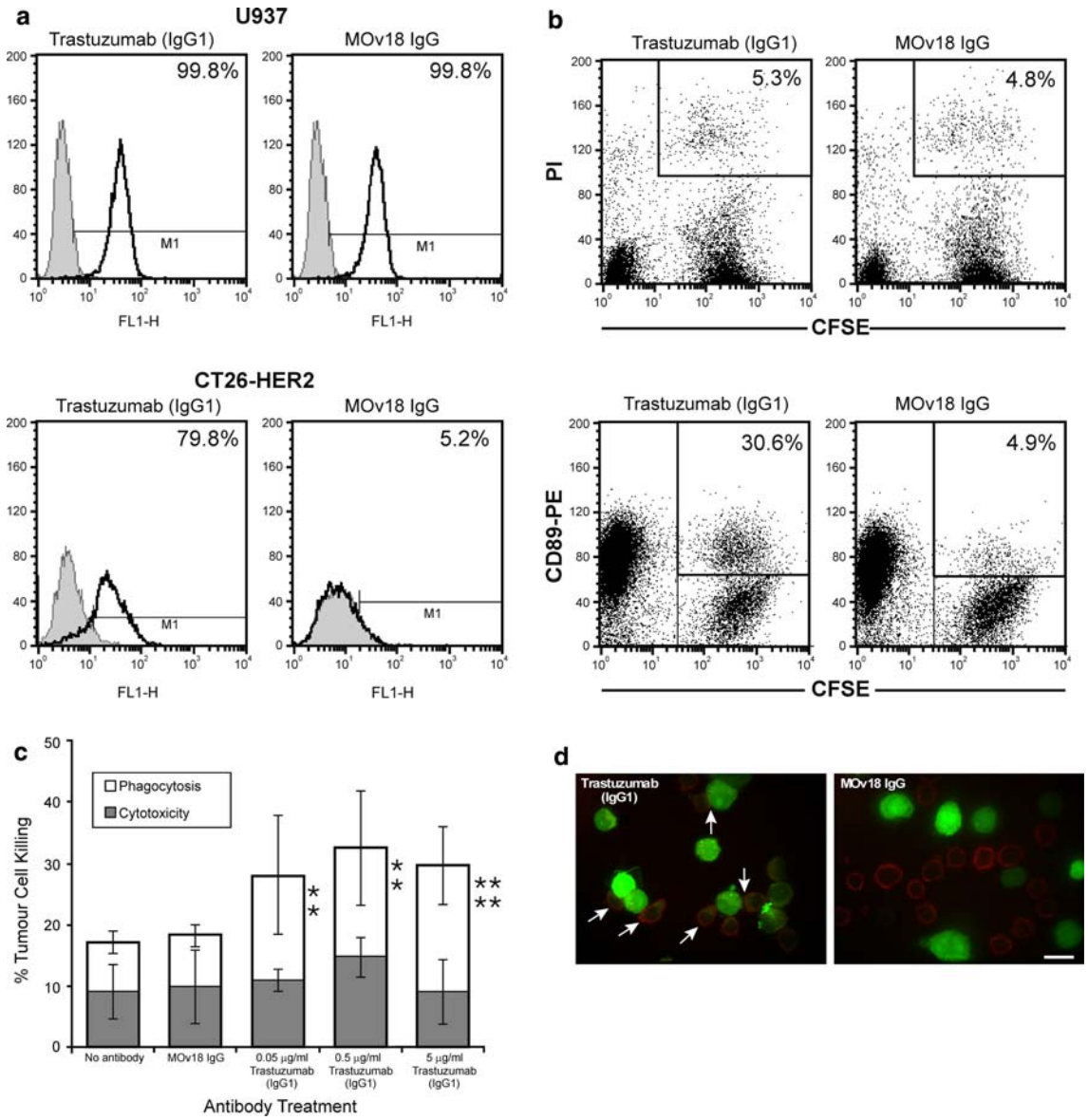

Fig. 3 **a** Flow cytometric analysis of binding to Fcγ receptor-expressing U937 monocytic cells for trastuzumab (IgG1) (*top left*) and the chimaeric MOv18 IgG antibody against the ovarian cancer antigen FBP (*top right*). Flow cytometric analysis of binding to the HER2/*neu* antigen on the surface of CT26-HER2/*neu* cells for trastuzumab (*bottom left*) and the chimaeric MOv18 IgG antibody against FBP (*bottom right*) (Monoclonal antibodies: *solid lines*; secondary antibody controls: *grey histograms*). **b** Trastuzumab-mediated killing of CT26-HER2/*neu* tumour cells by U937 monocytes: two-colour flow cytometric dot plots detected no ADCC but appreciable ADCP after 2.5 h in culture. CFSE-labelled tumour cells (*x*-axis) and dead tumour cells labelled with Propidium Iodide (PI) (*y*-axis), double-positive cells depict tumour cells killed by ADCC ($CFSE^+/PI^+$, *upper right boxes*, values are % ADCC) (*top*). CFSE-labelled tumour cells phagocytosed by U937 cells labelled with CD89-PE mAb (*y*-axis, *upper left*), double positive cells, $CFSE^+/PE^+$ (*upper right boxes*, values are % ADCP) (*bottom*). **c** Quantification of trastuzumab-mediated CT26-HER2/*neu* tumour cell killing by U937 monocytes after 2.5 h using the ADCC/ADCP assay. Cytotoxicity: *black bars*; phagocytosis: *white bars*. Results are means ± SD of six independent experiments. Significance of values compared to samples given MOv18 IgG (*top*) or no antibody (*bottom*) by the Student's *t* test: $^{n/s}P > 0.05$, $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. **d** Representative confocal fluorescence images of CT26-HER2/*neu*-U937 interactions potentiated by trastuzumab. CFSE-stained CT26-HER2/*neu* tumour cells (*green*) and CD33-APC labelled U937 cells (*red*) combined at an original E:T ratio of 2:1 and incubated for 3 h in culture. U937 cells (*red*) given trastuzumab IgG (*left*) showed enhanced contact with tumour cells (*green*) and phagocytosis of tumour cells (*green* CFSE inside U937 monocytes, *white arrows*). Neither effector-target cell contact nor phagocytosis was observed when cells were incubated with control MOv18 IgG antibody (*right*). Original magnification ×63 (*scale bar* 15 μm)

HER2/*neu* on the surface of CT26-HER2/*neu* cells. Trastuzumab IgE bound to Fcε receptors expressed on the surface of U937 monocytic cells (53.6% of cells) (Fig. 4a, upper left), in a manner indistinguishable from the positive control MOv18 IgE (60.6% of cells) (Fig. 4a, upper right). Lower levels of trastuzumab IgE binding to Fcε receptors,

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Table 3 Microscopic Measurements of CT26-HER2/*neu*: U937 Cell Interactions

Antibody	E:T contact (mean ± SD) (%)	CT26-HER2/*neu* phagocytosis (mean ± SD) (%)
Trastuzumab (IgG1)	24.5 ± 10.5**	20.43 ± 9.2***
MOv18 IgG	7.9 ± 11.2	3.5 ± 5.9
Trastuzumab IgE	29.2 ± 13.9***	4.7 ± 5.8 $^{n/s}$
MOv18 IgE	5.1 ± 5.2	2.2 ± 5.1

Data were collected by counting effector: target cell contact or target cell phagocytosis per microscopic field at a magnification of 40× and percentage values were calculated. Mean values were calculated from ten microscopic fields for each condition and are shown ± SD. Student's *t* test was used to generate significance of values compared to samples given the corresponding MOv18

$^{n/s}$ $P > 0.05$; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$

compared to binding of trastuzumab binding to Fcγ receptors, are consistent with lower expression of IgE receptors on U937 cells (Table 2) and our previous findings that MOv18 IgE bound to only a fraction of FcεRI receptors on these cells [30]. Trastuzumab IgE also recognised the HER2/*neu* receptor since it bound to 89.3% of CT26-HER2/*neu* cells (Fig. 4a, bottom left), similarly, to trastuzumab (91.5% cells, Fig. 4a, bottom middle), whilst only background binding of trastuzumab IgE was detected in untransfected CT26 cells, which do not express the HER2/*neu* receptor (5.2%) (Fig. 4a, bottom right). These data confirm the specificity of the engineered IgE antibody for the human HER2/*neu* antigen as well as for the Fcε receptors expressed on the surface of the monocytic cells.

Assessments of the functional properties of trastuzumab IgE

Monocytic cells and IgE-mediated tumour cell killing

We wished to assess whether our engineered trastuzumab IgE was biologically active and thus sought to characterise its biological properties using two functional assays. One related to the ability of this antibody to mediate tumour cell targeting and killing by human effector cells and was assessed using our three-colour flow cytometric ADCC/ADCP assay. As done above for trastuzumab, the CT26-HER2/*neu* cells were used as tumour targets and human U937 monocytes were employed to provide effector cells. Using this method, we observed that incubation of CT26-HER2/*neu* and U937 cells with trastuzumab IgE was associated with increased tumour cell death by cytotoxicity (ADCC) (Fig. 4b, c). This was evident by the increased population of CFSE +/PI + tumour cells in samples incubated with trastuzumab IgE (Fig. 4b, upper right, top right boxes for CFSE +/PI + tumour cells), compared to those with the control MOv18 IgE (18.7 vs. 4.6%; Fig. 4b, upper left). In contrast to trastuzumab, the neither the trastuzumab nor the MOv18 IgE enhanced the phagocytosis of tumour cells, as seen in the double positive CFSE +/PE + cell population (4.7 vs. 4.3%; Fig. 4b, bottom panel, top right boxes for CFSE +/PE + tumour cells). These results also confirmed that phagocytic killing by trastuzumab and U937 monocytes, measured by the ADCC/ADCP assays (Fig. 3), were a result of Fcγ receptor functions of this antibody rather than non-specific uptake of killed tumour cells.

Therefore, flow cytometric ADCC/ADCP assay measurements confirmed that trastuzumab IgE, at an optimal concentration of 0.5 μg/mL mediated significant levels of ADCC of CT26-HER2/*neu* cells by the monocytic cells (Fig. 4c). Levels of ADCC increased by ~10% above those of the MOv18 IgE, tested at the same concentrations, and the no antibody controls (Fig. 4c and Supplementary Table II, Supplementary Data). Statistically significant levels of ADCP were not measured in this assay system ($P > 0.05$; Supplementary Table II, Supplementary Data). These data suggest that trastuzumab IgE mediates tumour killing by a mechanism different from trastuzumab, directing monocytes to act in ADCC instead of ADCP against the tumour cells.

Interestingly, the antibody concentration (0.5 μg/mL) required to achieve maximum tumour cell killing by monocytes in these assays was the same for IgG and IgE (Fig. 3c, Fig. 4c and Supplementary Table II, Supplementary Data). Furthermore, when compared directly in ADCC/ADCP assays, the two IgE antibodies mediated similar levels of total tumour cell killing (35% of tumour cells by IgE vs. 36% of tumour cells by IgG; Table 4). Our data show that IgE is as effective as IgG in recruiting monocytes to kill tumour cells in vitro and warrant further studies to compare the anti-tumour effector cell functions of these antibodies in vivo.

Trastuzumab IgE-mediated interactions of monocytic and tumour cells

The tumour-targeting activities of trastuzumab IgE measured in the ADCC/ADCP assays were studied by confocal microscopical imaging (Fig. 4d). CT26-HER2/*neu* cells were pre-labelled with CFSE (green), incubated with U937 cells, combined with antibodies for 3 h on glass chamber slides, and U937 cells were labelled with anti-CD33-APC (red). In samples incubated with trastuzumab IgE, contact between CT26-HER2/*neu* tumour cells and U937 monocytic cells, was clearly evident, and two or more monocytic cells were frequently observed in contact with or in close proximity to a single tumour cell (Fig. 4d, left; white arrows). However, in contrast to our observations with

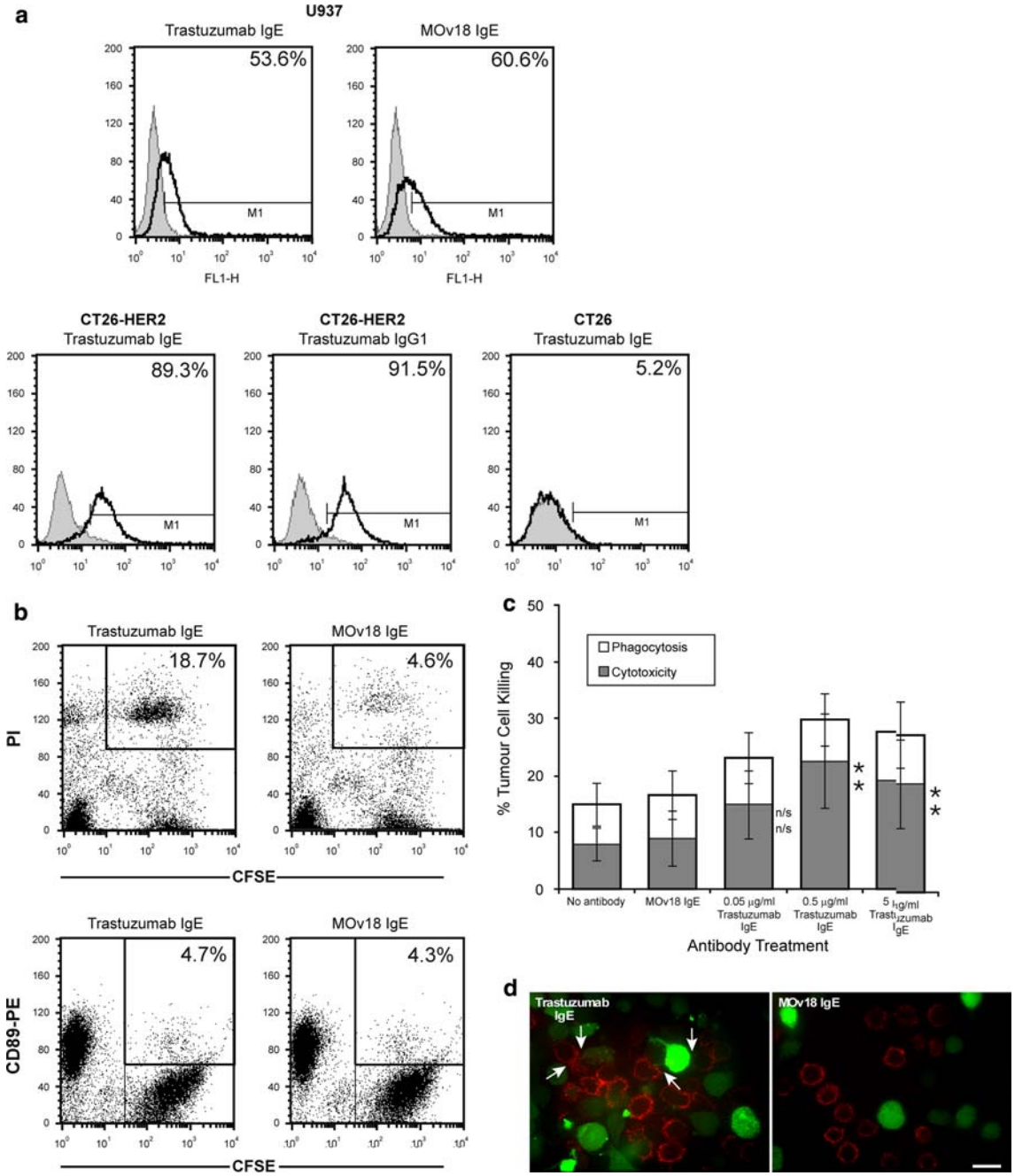

Fig. 4 **a** Flow cytometric analysis of binding to IgE receptor-expressing U937 monocytic cells for trastuzumab IgE (*top left*) and the chimaeric MOv18 IgE antibody against the ovarian cancer antigen FBP (*top right*). Flow cytometric analysis of trastuzumab IgE (*bottom left*) and trastuzumab (IgG1) antibody (*bottom middle*) binding to the HER2/*neu* antigen on the surface of HER2/*neu*-expressing CT26-HER2/*neu* cells, and lack of trastuzumab IgE binding to HER2/*neu*-negative CT26 tumour cells (*bottom right*). (Monoclonal antibodies: *solid lines*; secondary antibody controls: *grey histograms*). **b** Trastuzumab IgE-mediated killing of CT26-HER2/*neu* tumour cells by U937 monocytes: two-colour flow cytometric dot plots detected ADCC but no ADCP after 2.5 h in culture. CFSE-labelled tumour cells (*x*-axis) and dead tumour cells labelled with Propidium Iodide (PI) (*y*-axis), double-positive cells depict tumour cells killed by ADCC ($CFSE^+/PI^+$, *upper right boxes*, values are % ADCC) (*top*). CFSE-labelled tumour cells phagocytosed by U937 cells labelled with CD89-PE mAb (*y*-axis) are double positive $CFSE^+/PE^+$ cells (*upper right boxes*, values are % ADCP) (*bottom*). **c** Quantitation of trastuzumab IgE-mediated CT26-HER2/*neu* tumour cell killing by U937 monocytes after 2.5 h using the ADCC/ADCP assay, at different antibody concentrations. Cytotoxicity: *black bars*; phagocytosis: *white bars*. Results are mean ± SD of five independent experiments. Significance of values compared to samples given MOv18 IgE (*top*) or no antibody (*bottom*) by the Student's *t* test: $^{n/s}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. **d** Typical confocal fluorescence images of CT26-HER2/*neu*-U937 interactions potentiated by trastuzumab IgE. CFSE-stained CT26-HER2/*neu* tumour cells (*green*) and anti-CD33-APC labelled U937 cells (*red*) combined at an original E:T ratio of 2:1 and incubated for 3 h in culture. U937 cells (*red*) given trastuzumab IgE (*left*) exhibited enhanced contact with tumour cells (*green*) but no phagocytosis of tumour cells was observed (no *green* CFSE fluorescence detected inside U937 cells; *white arrows*). No effector-target cell contact was observed when cells were incubated with control MOv18 IgE antibody (*right*). Original magnification ×63 (*scale bar* 15 µm)

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Table 4 % Total tumour cell death by U937 monocytes and antibodies

Antibody	Tumour cell death ± SD ($n = 6$) (%)
No ab	12.5 ± 3.1
MOv18 IgG	18.5 ± 6.6
Trastuzumab (IgG1) 0.5 μg/mL	36.1 ± 2.2 (***)(***)
MOv18 IgE	19.8 ± 3.2
Trastuzumab IgE 0.5 μg/mL	34.8 ± 3.5 (***)(***)(n/s)

Significance comparing trastuzumab (IgG1) and trastuzumab IgE to samples given no antibody (left brackets), MOv18 IgG/IgE (middle brackets) and significance comparing trastuzumab (IgG1) to trastuzumab IgE (right bracket)

Comparisons by the Student's t test: (n/s)$P > 0.05$; (*)$P < 0.05$; (**)$P < 0.005$; (***)$P < 0.0005$

trastuzumab, trastuzumab IgE did not appear to enhance phagocytosis, since no green tumour material was observed inside the red U937 cells (Fig. 4d, left). As expected, rare contact was observed with the control antibody MOv18 IgE, examined in the same culture conditions (Fig. 4d, right). Measurements of interactions between effector and target cells (Table 3) showed that trastuzumab IgE mediated enhanced effector: target cell contact (29.2% of tumour cells) after 3 h, compared to 5.1% contact in samples given MOv18 IgG. A very small proportion of the U937 monocytes in contact with tumour cells contained tumour cell material (4.7% of tumour cells), suggesting little tumour cell phagocytosis was mediated by trastuzumab IgE. Microscopic observations and measurements of effector: tumour cell interactions are in agreement with our ADCC/ADCP assays and are consistent with a role for trastuzumab IgE in mediating tumour cell death by cytotoxicity.

Trastuzumab IgE activity in mast cell degranulation assays

The second functional assay examined the capacity of trastuzumab IgE to stimulate mast cells by way of the high-affinity IgE receptor FcεRI, and trigger their degranulation. This is a test of the potency of IgE to activate these effector cells, and occurs only following cross-linking of FcεRI-bound IgE by multivalent antigen, by anti-human IgE polyclonal antibodies or by antigen-expressing target cells. We used a previously established system, designed to evaluate the functional activities of IgE antibodies [48]. The assay utilises the rat basophilic mast cell line RBL-SX38, transfected to express all four subunits ($\alpha\beta\gamma_2$) of human FcεRI [48]. Flow cytometric evaluations confirmed that trastuzumab IgE bound to cell surface FcεRI of the RBL-SX38 cells (45.3% of cells), similar to the previously characterised chimaeric MOv18 IgE (Fig. 5a). To assess the ability of trastuzumab IgE to cause degranulation of RBL-SX38 cells, we measured β-hexosaminidase release upon cell stimulation and cross-linking by tumour cells (Fig. 5b). Mast cells alone and mast cells stimulated with trastuzumab IgE in the absence of cross-linking by anti-IgE antibody, triggered minimal mast cell degranulation [54]. Mast cells stimulated with trastuzumab IgE in the presence of anti-IgE antibody triggered a strong degranulation response (~40%), compared to negligible degranulation measured with controls.

We also examined whether the engineered trastuzumab IgE cross-linked by HER2/*neu*–expressing tumour cells was capable of stimulating mast cells in an antigen-dependent manner. Trastuzumab IgE induced strong degranulation of RBL-SX38 cells following stimulation with CT26-HER2/*neu* cells, which express HER2/*neu,* whilst untransfected CT26, which do not express HER2/*neu*, did not potentiate β-hexosaminidase release (Fig. 5b). Furthermore, CT26-HER2/*neu* tumour cells with trastuzumab IgE potentiated significant β-hexosaminidase release that was decreased proportionally to the decreasing number of tumour cells per sample. These results clearly demonstrate the functional activity of trastuzumab IgE-CT26-HER2/*neu* cells in triggering mast cell activation and mediator release, and confirm that the IgE possesses biological activities that could be specifically directed against HER2/*neu*-expressing tumour cells in cancer patients.

Trastuzumab IgE targeting of SKBR3 breast cancer cells

ADCC/ADCP assays using the human breast adenocarcinoma cell line SKBR3, which naturally express the HER2/*neu* antigen, confirm that trastuzumab and trastuzumab IgE can focus U937 effector cells to kill SKBR3 cells (Fig. 6). As with CT26-HER2/*neu* cells (Figs. 3, 4), trastuzumab acted in ADCP of tumour cells (Fig. 6a) whilst trastuzumab IgE killed by ADCC (Fig. 3b). These findings demonstrate the functional properties of trastuzumab IgE focusing effector cell functions against native HER2/*neu*-expressing breast tumour cells.

Since trastuzumab (IgG1) can potentiate anti-tumour effects by blocking hetero-dimerisation of HER2/*neu* receptors with other HER family members on the surface of breast cancer cells, switching off tumour cell growth signals [8, 9], the anti-proliferative properties of the engineered trastuzumab IgE were examined using the MTS cell viability assay (Fig. 6c). Neither trastuzumab nor trastuzumab IgE had any anti-tumour growth effects after 4 h incubation with SKBR3 cells (99.3 and 98.6% viability for IgG and IgE, respectively). Decreased tumour cell viability was measured after 24 h (88.3 and 83.6% for IgG and IgE, respectively) and more prominent effects were measured after 48 h exposure of tumour cells to the antibodies (72.2 and 64.0% for IgG and IgE, respectively). Control MOv18 IgG and MOv18 IgE antibodies did not affect SKBR3 cell viability (mean values ranging from 93.0 to 103.8% viability).

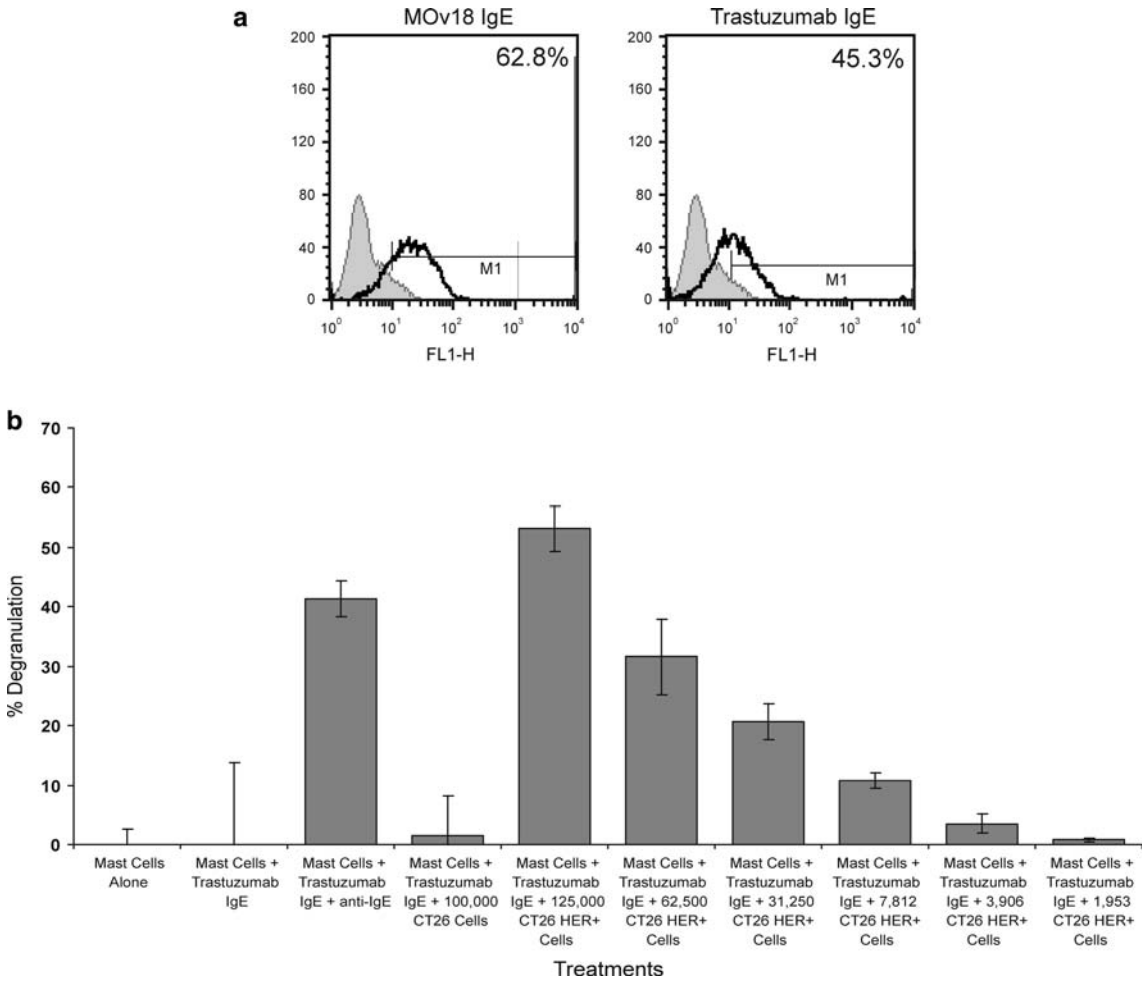

Fig. 5 **a** Flow cytometric analysis of MOv18 IgE (*left*) and trastuzumab IgE (*right*) binding to human FcεRI receptor-expressing RBL-SX38 cells. (Monoclonal antibodies: *solid lines*; secondary antibody controls: *grey histograms*). **b** Effects of trastuzumab IgE cross-linking on mast cell degranulation. Cells alone or sensitised with trastuzumab IgE, CT26 tumour cells or CT26-HER2/*neu* tumour cells at different concentrations. Trastuzumab IgE was cross-linked with anti-IgE polyclonal antibody to confirm its mast cell degranulation activity. Degranulation was monitored by β-hexosaminidase activity released into culture supernatants in all experiments. Data are mean ± SD of three measurements in a representative of three different experiments with similar results

These data strongly suggest that trastuzumab IgE possesses similar properties to trastuzumab in blocking tumour growth after 24 and 48 h in culture.

Discussion

In our previous studies, we reported the anti-tumour activities of MOv18 IgE, a chimaeric antibody against the ovarian tumour-associated antigen FBP, compared to the corresponding antibody of the IgG class [29–32, 49]. Our studies now form part of a growing body of evidence suggesting that IgE antibodies may have a role in cancer therapy [21–23, 27, 28, 33–35, 55]. The emergence of the human epidermal growth factor receptor HER2/*neu* as a well-validated target for cancer therapeutics [1–4] and the well-documented but circumscribed success of the humanised anti-HER2/*neu* IgG1 antibody trastuzumab, approved for the treatment of breast cancer [5–7], rendered the proposition to produce an IgE equivalent moiety timely and relevant in the field of biological therapeutics for cancer. For this, we have engineered a humanised trastuzumab IgE antibody and here we describe the binding characteristics and biological properties of this molecule.

The kinetics of antigen binding and cell binding data suggest that trastuzumab IgE possesses HER2/*neu* binding properties that are similar to those observed and measured for trastuzumab (Table 1; Figs. 2, 3, 4). Similar to other well-characterised IgE antibodies (MOv18 IgE and NIP IgE), trastuzumab IgE bound to its high-affinity receptor FcεRI as expected (Table 1; Figs. 2, 4). Furthermore, using functional assays, this study demonstrates the biological activity of trastuzumab IgE antibody in directing effector cells to target tumour cells in a tumour antigen-specific manner (Figs. 4, 5).

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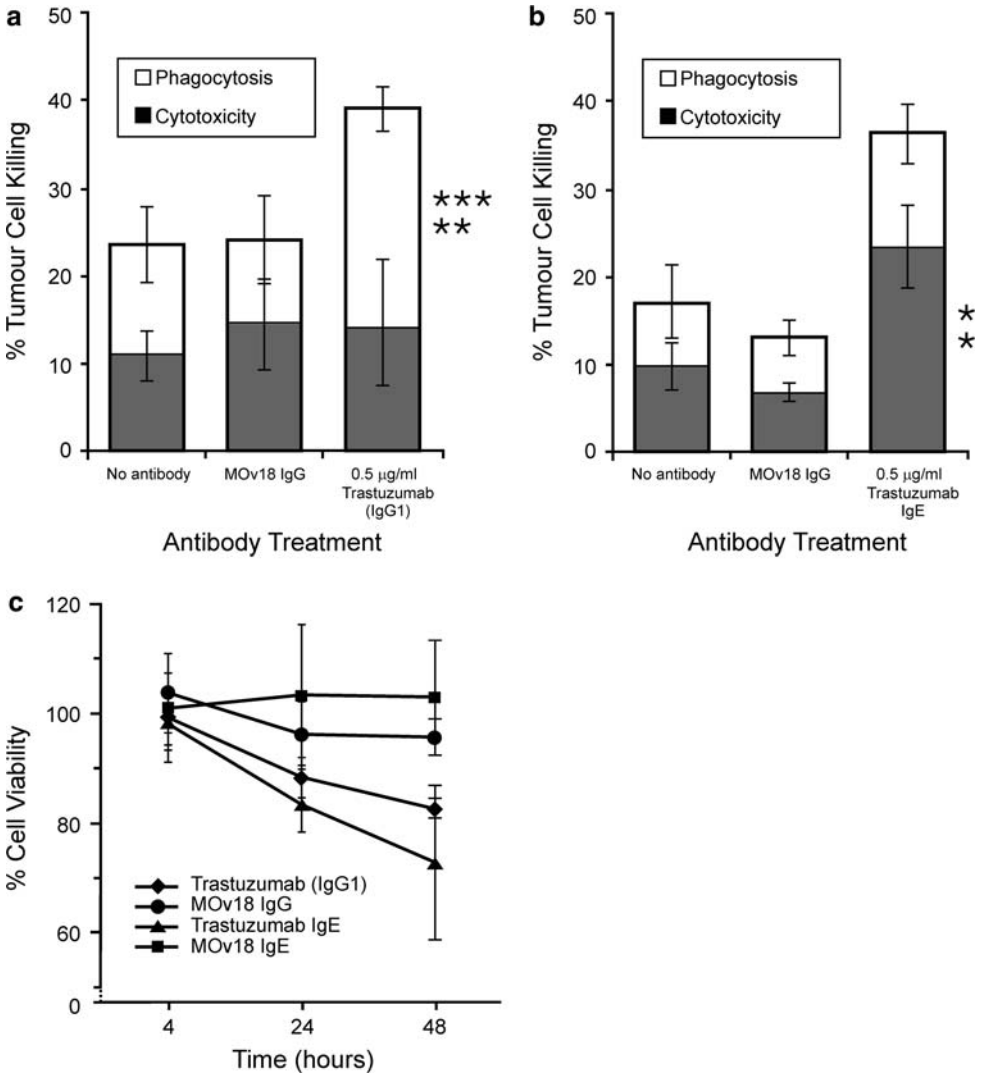

Fig. 6 **a** Quantitation of trastuzumab (IgG1)-mediated tumour cell killing of SKBR3 human breast tumour cells by U937 monocytes after 2.5 h using the ADCC/ADCP assay. **b** Quantitation of trastuzumab IgE-mediated tumour cell killing of SKBR3 human breast tumour cells by U937 monocytes after 2.5 h using the ADCC/ADCP assay. Cytotoxicity: *black bars*; phagocytosis: *white bars*. Results are means ± SD of 5 independent experiments. Significance of values compared to samples given MOv18 IgG/IgE (*top*) or no antibody (*bottom*) by the Student's *t* test: $^{n/s}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. **c** Cell viability assays (MTS) demonstrating levels of susceptibility of SKBR3 breast cancer cells to trastuzumab (IgG1), trastuzumab IgE, MOv18 IgG and MOv18 IgE antibodies at 4, 24 and 48 h in culture. Each data point represents mean % cell viability ± SD ($n = 4$)

Using our ADCC/ADCP assays and microscopic images, we made a number of observations relating to the effector functions of trastuzumab (IgG1) and trastuzumab IgE. Trastuzumab triggered appreciable levels of monocyte-mediated tumour cell phagocytosis by human monocytic cells (Figs. 3b, c, 6a), also clearly observed in confocal images by the presence of tumour cell material ingested by monocytic cells in contact with tumour cells (Fig. 3d; Table 3). Whilst the signalling and tumour growth arrest activities of trastuzumab and their role in its clinical efficacy have been the subject of many studies, the anti-tumour mechanisms relating to effector cell functions of trastuzumab have been less extensively investigated [7, 8, 10–14]. ADCC is a known anti-tumour mechanism of trastuzumab [11, 13, 14]. ADCP is not a widely described function attributed to trastuzumab, but our data are consistent with ADCP as a potential anti-tumour mechanism for trastuzumab with human macrophages, as reported by Lazar et al. [12].

Surprisingly, no trastuzumab-dependent monocyte-mediated cytotoxicity (ADCC) of tumour cells was detected above that seen in the controls in our ADCC/ADCP assays. Using our ADCC/ADCP assay to simultaneously measure the contributions of ADCC and ADCP [30, 31, 49], we detected only ADCP killing of tumour cells by trastuzumab. There are several possible explanations for the discrepancy between the present results and earlier reports. (1) Previous assays on the effector cell functions of trastuzumab, in combination with either unfractionated or purified human effector cell populations, did not assess the contributions of ADCP in the death of tumour cells [11–14]. It is therefore possible that ADCP-mediated tumour cell death may have been scored as ADCC if the assays measured total loss of tumour cells; (2) U937 monocytes express FcγRI and FcγRII, but very low levels of FcγRIII (Table 2), and thus it is possible that the absence of appreciable levels of FcγRIII may result in low levels of ADCC [18]; (3) The previously reported tumour cell trastuzumab ADCC

may have been mediated by other IgG receptor-bearing effector cells in peripheral blood lymphocytes, such as NK cells and neutrophils, as previously shown [11–14].

When compared to the FBP-specific MOv18 IgE antibody in the same assay, trastuzumab IgE activated monocytic effector cells to exhibit significantly enhanced contact with tumour cells and to effectively kill HER2/*neu*-expressing tumour cells (Fig. 4d; Table 3). Trastuzumab IgE directed monocytes to kill HER2/*neu*-transfected tumour cells and also tumour cells naturally-expressing the HER2/*neu* antigen by ADCC (cytotoxicity), a mechanism clearly different from that of the anti-tumour mechanism employed by trastuzumab in the same assay system (Figs. 3, 4, 6). This may reflect the specific binding of trastuzumab IgE to the high-affinity receptor FcεRI. Evidence presented in our previous studies on the mechanism of MOv18 IgE monocyte-mediated tumour cell killing, suggested that binding of IgE to its high-affinity receptor FcεRI on monocytes triggered tumour cell death by a cytotoxic mechanism, whilst binding to the low-affinity receptor CD23 resulted in tumour cell death by ADCP. U937 monocytes express low levels of CD23 (~2,200 molecules/cell, Table 2) and, as observed in our studies with MOv18 IgE, these receptor levels are too low to mediate ADCP of tumour cells [30]. The function of trastuzumab IgE effected through CD23 remains to be explored and may reveal additional tumour cell killing properties of the trastuzumab IgE.

Our assays clearly indicated that the levels of tumour cell killing mediated by trastuzumab IgE were equivalent to those by trastuzumab (IgG) at the same optimal doses and in the same assay system (Figs. 3, 4, Table 4). This was true despite the relatively low levels of IgE binding on the surface of U937 cells, compared to IgG (Figs. 3a, 4a), in agreement with previous findings [30] suggesting the potency of IgE-mediated effector functions [20–23, 27, 28, 35, 56]. IgE has much higher affinity for FcεRI ($Ka = 10^{10}\,M^{-1}$) than IgG1 has for any of its three Fcγ receptors, especially for FcγRIII ($Ka = 10^{5}\,M^{-1}$), the main receptor associated with tumour cell killing [17, 18, 57]. The relatively high affinities of the IgE-FcεRI interaction may compensate for the lower binding of IgE on U937 monocytes, resulting in comparable levels of tumour cell killing. These data clearly indicate the biological function of trastuzumab IgE in focusing monocytic cells to kill HER2/*neu*-expressing tumour cells as effectively as, but through a mechanism different from, trastuzumab.

The antibody concentration (0.5 μg/mL) required to achieve maximum tumour cell killing by ADCC/ADCP was the same for IgG and IgE in our in vitro assays (Figs. 3, 4). In addition, our cell viability data clearly show that trastuzumab IgE, at concentrations found optimal for effector cell responses (0.5 μg/mL), maintains the ability to mediate tumour cell growth arrest over a period of 48 h in culture and at levels similar to those measured for trastuzumab (Fig. 6c). The concentration found optimal for the in vitro effector cell functions of trastuzumab IgE was ten-fold lower than our previously reported optimal concentrations required for MOv18 IgG and IgE-mediated (5 μg/mL) killing of ovarian tumour cells in equivalent in vitro assays [30–32]. This may suggest that lower levels of trastuzumab IgE may be required for in vivo activities compared to those used for MOv18 IgG and MOv18 IgE studies. The IgE responses measured and observed in our assays support the conclusion that trastuzumab IgE functions with similar potency, but through mechanisms different from those of trastuzumab in vitro, warranting further exploration of this engineered antibody in more clinically relevant models. It is possible, however, that trastuzumab IgE may be more effective than trastuzumab (IgG1) in vivo for the reasons cited in the Introduction or that a combination of trastuzumab and trastuzumab IgE could have potential synergistic anti-tumour effects.

We also assessed the potential of the engineered trastuzumab IgE to activate other potent IgE receptor-bearing effector cells. For this, we have utilised a functional assay that exemplifies the unique properties of IgE to generate and enhance immune effector functions that can be targeted against cancer cells. Trastuzumab IgE bound to the human FcεRI $\alpha\beta\gamma 2$ receptor on the surface of mast cells can be cross-linked by HER2/*neu*-expressing tumour cells to trigger mast cell degranulation in an antigen-specific manner (Fig. 5). Tissue mast cell degranulation is a known biological property of the IgE antibody class. Mediators released by degranulated mast cells initiate and potentiate effector cell recruitment to the site of tumour antigen challenge, which can be expected to lead to activation and stimulation of recruited and locally present effector cells to act in the ADCC and ADCP of tumour cells. Mast cell activation by IgE may thus serve as a potential trigger of a strong, local, tumour antigen-specific IgE immune response against cancer.

This is the first study describing the properties of an engineered trastuzumab IgE. Based on the considerable evidence pointing to a number of advantages that IgE may have in tumour cell surveillance and killing, compared to IgG, our work points to the importance and value of further research to investigate the efficacy and mechanisms of action of tumour antigen-specific antibodies of different classes, in particular IgE and may help to realise the full potential of antibodies for immunotherapy of cancer.

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CHAPTER VI

Summary

Breast cancer is the most common malignancy in women worldwide. As cancer is a disease with increasing prevalence, broad research is going to develop innovative, cost-effective and more patient-friendly therapies. Complementary to the standard treatment of breast cancer, radiotherapy and chemotherapy, therapeutic approaches have focused on the involvement of the immune system. Thereby, the passive application of monoclonal antibodies, such as trastuzumab (Herceptin®), targeting the tumour antigen HER-2 on breast cancer cells in a highly specific manner became state of the art therapy. Nevertheless, several disadvantages and side effects are known.

As our aim was to improve passive immunotherapy with monoclonal antibodies based on IgE antibodies, we applied two different approaches: on the one hand we focused on the mimotope strategy, and on the other hand we changed the subclass of the monoclonal antibody trastuzumab from IgG to IgE.

The mimotope approach is well-known in the field of allergology and cancer. A mimotope is generated by the phage display technology representing an epitope-mimic of the targeted antigen. Active immunization with a mimotope, generated with the anti-HER-2 antibody trastuzumab in a biopanning procedure, leads to the production of trastuzumab-like antibodies with similar biological functions. As previous studies in our group have focused on the anti-tumour function of the mimotope-induced IgG antibodies, we were interested in the tumouricidal effect of mimotope-induced IgE antibodies.

Depending on the route of immunization different antibody subclasses are produced. In CHAPTER III of this thesis we could show that via oral immunization under acid suppression IgE antibodies with high affinity to the antigen are preferable induced. In this study we used

the food allergen ovalbumin (OVA) as a model antigen. We have reported that patients treated with acid-suppression medication due to dyspeptic disorders produce specific IgE antibodies against common dietary antigens. We could confirm that the intake of anti-acid medication results in an elevation of the gastric pH with the consequence that digestion-labile proteins remain stable, become allergenic and therefore enhance the induction of antigen-specific IgE antibodies. We performed comparison studies of different concentrations of OVA and different routes of administration. We found out that feedings of OVA alone did not result in IgE production at any concentration whereas oral administration of the lowest dose of OVA (0.2 mg) under acid-suppression resulted in specific and biologically functional IgE and an increase of the Th2 cytokine IL-5.

Based on this knowledge we combined the mimotope strategy and the oral immunization scheme under acid suppression in an anti-cancer study (CHAPTER IV). In a mouse study, active vaccination of the HER-2 mimotope via the oral route under simultaneous hypoacidic gastric conditions resulted in HER-2-specific IgE production *in vivo*. To answer the question if these IgE antibodies are antigen-specific and biologically active we performed a RBL assay. The IgE antibodies binding to FcεRI on RBL cells were incubated with HER-2 overexpressing tumour cells. Recognition and binding of IgE to HER-2, led to cross-linking of FcεRI and degranulation of the RBL cells. This reaction was antigen-specific, because incubation with a HER-2 negative control cell line did not result in cross-linking and β-hexosaminidase release. In a next step the cytotoxic potential of the RBL cells as effector cells was investigated in an ADCC assay with HER-2 overexpressing tumour cells as target cell line. Mimotope-induced IgE antibodies led to lysis of 20% to 100% of tumour cells in a highly specific manner. This study clearly demonstrated the active induction of tumour-specific IgE led to killing of the tumour cells by cross-linking of FcεRI on potent effector cells, such as RBL cells.

Furthermore, to directly compare the anti-tumour effects of IgG and IgE we generated an IgE analogue of trastuzumab as described in CHAPTER V. To prove whether the newly engineered IgE antibody recognizes HER-2 and the IgE receptors we performed kinetic studies using surface plasmon resonance analysis. Besides, the binding characteristic of trastuzumab IgG and IgE was investigated on the monocytic cell line U937 and on HER-2 overexpressing tumour cells by flow cytometry. These cell lines served in the subsequent killing experiment as effector and target cells. Our results confirmed that trastuzumab IgE bound to HER-2 comparable to trastuzumab IgG. Moreover, trastuzumab IgE bound to the high affinity IgE receptor comparable to other control IgE antibodies. The next step was to assess the biological activity and the comparison of trastuzumab IgE and IgG in respect of their tumor cell killing mechanism in a three-colour flow cytometric ADCC/ADCP assay. We could demonstrate that IgE is as effective as IgG in recruiting monocytes to kill tumour cells *in vitro.* In contrast to an unspecific control IgE antibody we observed that incubation of trastuzumab IgE, U937 cells and HER-2 positive target cells was associated with increased tumour cell death by cytotoxicity (ADCC), whereas trastuzumab IgG killed by phagocytosis (ADCP). In a RBL assay, incubation of trastuzumab IgE with HER-2 overexpressing cells led to mast cell activation and mediator release. Furthermore, we were interested in the anti-proliferative properties of trastuzumab IgE compared to its IgG analogue. Therefore we performed a cell viability assay and observed that after 24 h and 48 h incubation of IgE and IgG growth of HER-2 positive tumour cells was inhibited with a similar percentage.

From the results presented in this thesis we conclude that there is evidence that IgE antibodies directed against tumour antigens, either applied in an active or passive manner, are efficient in mediating tumour cell killing.

Contribution to publications

CHAPTER III

Dose-dependent food allergy induction against ovalbumin under acid-suppression: A murine food allergy model

The author of this PhD thesis took part in all steps of the oral immunization regime of BALB/c mice and in the experimental steps of the read out of the murine sera, which was done by ELISA and RBL assays. The author also contributed to the type I skin tests and the spleen preparation for cytokine measurement of the mice. Further, the author contributed to the manuscript preparation and the proof reading of the manuscript.

CHAPTER IV

Active induction of tumour-specific IgE antibodies by oral mimotope vaccination

In this study the author contributed mainly in the preparation of the cell culture (SKBR-3 and A431 and RBL cell lines) and all cellular assays. The author performed the RBL assay and the cytotoxicity assay showing the ADCC potential of IgE antibodies.

CHAPTER V

Characterisation of an engineered trastuzumab IgE antibody and effector cell mechanisms targeting HER-2/neu-positive tumour cells

As this publication was a multidisciplinary study between the Medical University of Vienna, King's College London and the Division of Surgical Oncology of UCLA the coordination of all experiments was an essential part. The author contributed to the study design and took part in the correspondence between the involved institutes, the scientific exchange and trouble shooting and conducted the material preparation and transfer from Vienna as well as proof reading of the manuscript.

3.2.2010

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Curriculum vitae

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Original articles

1. **Mimotope vaccination - from allergy to cancer.** Knittelfelder R, Riemer AB, Jensen-Jarolim E. Expert Opin Biol Ther. 2009 Apr;9(4):493-506

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