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IgE-based cancer immunotherapy



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Declaration

I declare that this thesis has been composed by myself, Natasa Vukovic, and that the work submitted is my own, unless otherwise clearly stated. The work included in this thesis has not been submitted for any other degree or professional qualification. In the thesis appendix the articles that resulted from the work carried out during my PhD are provided.

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Abstract

Anti-tumour effect of monoclonal antibodies (mAbs) can be achieved through different mechanisms in which both target engagement with Fab arm and Fc-mediated recruitment of effector immune cells play an important role. As different antibody isotypes bind to different FcRs on immune cells and also differ in their potential to activate the complement system, they can skew the immune reaction towards different effector functions. Therefore, isotype selection can be of critical importance for treatment efficacy. For instance, the IgE isotype has shown superior tumour control to traditionally used IgG in several preclinical models and represents a promising alternative. This thesis offers a further evaluation of IgE therapy in cancer, by exploring different types of target antigens – surface, secreted, and intracellular. In addition, by conducting the experiments in wild-type mice with FcεRI expression limited to MCs and basophils, a particular focus was put on these cell types as less explored IgE effector cells in cancer settings.

Firstly, a **surface tumour antigen** was targeted, and the efficacy of three different antibody isotypes (IgG2a, IgG1 and IgE) was directly compared in a syngeneic mouse tumour model. To this end, anti-Thy1.1 antibodies were used to target subcutaneously transplanted B16-OVA-Thy1.1 cells. The results show that the anti-Thy1.1 antibody with an IgG2a isotype was superior to the IgG1 and the IgE isotype antibodies in controlling tumour growth in this setup. IgE did not show any effect on tumour growth. Since MCs and basophils were mainly localised at tumour edges, we believe there was a poor interaction between them and tumour cells. Thus, targeting a surface tumour antigen does not seem optimal for unleashing IgE downstream effects.

Next, the effects of IgE treatment targeting a **secreted tumour antigen** in combination with OT-1 adoptive cell transfer were studied in the B16-OVA model. Here, two different IgE antibodies targeting a secreted antigen (OVA) were used to achieve effector cell degranulation. My data shows that IgE-induced effects abolished the protective effect of OT-1 cells, suggesting that IgE induces unfavourable changes in the TME.

Finally, I studied how the polyclonal immune response raised by immunising mice against an **intracellular tumour antigen** under type-2 immune skewing conditions affected the tumour

development in B16-OVA syngeneic model and in the spontaneous prostate tumour model (TRAMP mice). Although the data are not completely conclusive, there are indications that type-2 immune skewing in combination with CD8⁺ T cell adoptive cell transfer has protective effects. This finding indicates that type-2 immune skewing made the TME less immune-suppressive, allowing the transferred CD8⁺ T cells to exert their cytotoxic effect more efficiently.

In addition, three manuscripts that resulted from my PhD work are provided in the thesis appendix. **First**, we published a review article discussing the use of different antibody isotypes for tumour targeting antibodies, checkpoint inhibitors and agonistic antibodies in cancer settings. **Second**, the method for IgE purification with thiophilic interaction chromatography (TIC) was published. IgE purification is somewhat complicated, as standard methods used for IgG purification cannot be applied to IgE. Described TIC is relatively cheap, gentle in terms of pH and can be used to purify any IgE antibody, irrespective of its specificity or species of origin.

Third, we published a manuscript describing the development of the first human IgE bispecific molecules. I developed human IgE bispecific antibodies as a novel format that would combine the increased selectivity of bispecific antibodies with the potent downstream effector function of the IgE isotype. Both knobs-into-holes strategy and leucine zipper mediated molecule pairing resulted in fully functional bispecific IgE molecules. Importantly, bispecific IgE was superior to its bispecific IgG1 homologue in ADCC-mediated *in vitro* killing of tumour cells.

Taken together, the work described here contributes to our current understanding of IgE-mediated effects on tumour growth. Importantly, it focuses on greatly overlooked IgE effector populations in cancer – MCs and basophils, and gives insight on the different effects IgE can mediate through these cell types depending on the localisation of the targeted antigen and the type of exposure. This indicates that IgE based immunotherapy can lead to different outcomes depending on the context. In addition, my thesis offers important tools for further IgE research in terms of simplifying IgE purification and describing the human bispecific IgE formats for the first time.

Lay Summary

The immune system represents a complex net of organs, cells and mediators whose primary function is to fight infection. However, the immune system is also significantly involved in fighting cancer, as cancer cells are often recognised as abnormal and foreign to our bodies. One of the ways in which our immune system recognises and fights those ‘foreign’ cancer cells is via antibodies (immunoglobulins (Ig)) it produces. Today, we can also produce those antibodies in laboratories and give them as treatment. Nevertheless, there are five different classes of antibodies, and all of them have different responsibilities. For example, all the therapeutic antibodies currently belong to the IgG class. However, the IgE class, which is the one that is involved in allergic reactions, has shown some promising results as a cancer treatment as well. Since the activation of our immune system during the allergic reaction can be extremely strong, if we could redirect it, we could potentially use the immune system to destroy cancer cells.

Thus, my thesis focuses on finding an optimal way of provoking such an allergic reaction inside the tumour. I studied this question in mouse models by using IgE antibodies that recognise either a molecule present on the surface of the tumour cell (chapter 2) or inside the tumour cell (chapter 4), or that is released (secreted) from the tumour cell (chapter 3). Once the IgE finds that antigen, it activates immune cells, mainly MCs and basophils. Those cells then can affect tumour development in different ways.

In addition, I combined the IgE treatment with another important player of anti-tumour immunity – T cells. T cells have a great potential of destroying cancer cells. However, cancer cells are smart, and they often create an environment in which T cells are suppressed and inactive. Thus, I wanted to test if IgE treatment could revert that unfavourable environment and work together with T cells to fight cancer.

My results show that the localisation of the targeted tumour molecule greatly affects the outcome of IgE treatment. For example, when I used IgE against a molecule present on the **surface** of tumour cells, I did not observe any effect on tumour growth, either good or bad. On the other hand, IgE against a molecule **secreted** from tumour cells had a detrimental effect, as it made it even harder for T cells to control tumour growth. Finally, the results in

the last chapter indicate that IgE against a molecule present **inside** the tumour cell may have a beneficial effect. In this setup, T cells worked better and offered better control of tumour growth.

In addition, a part of my work that is already published is given in the **appendix**. First, a paper that reviews how different antibody classes should be selected for cancer therapy. Second, a paper that describes a simple, gentle and universal IgE purification method. Finally, a paper in which I describe the development of the first human bispecific IgE antibody formats. Antibodies have the shape of the letter 'Y', and with both arms they recognise the same molecule. However, bispecific antibodies are engineered so that each arm recognises a different molecule. In this way, by binding to two different tumour molecules, bispecific antibodies are more strictly directed to tumours and have fewer chances of binding to healthy cells elsewhere in the body. Thus, the risk of side effects is minimised.

Taken together, the results presented in this thesis highlight the variety of effects that IgE can have on tumour development through MCs and basophils. The IgE effects depended on the localisation of its targets within the tumour cell. In that regard, the optimal outcome was achieved with IgE directed against a molecule inside the tumour cells, and not on the surface or secreted. In addition, I developed useful tools for further research of IgE antibodies – a method of IgE purification and a design of the first IgE bispecific antibodies.

Abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cell-mediated phagocytosis
ANGPT1	Angiopoietin 1 gene
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
A/I	Activating-to-inhibitory
BAT	Basophil activation test
BET	Basophil extracellular trap
β-hex	Beta-hexosaminidase
BMMC	Bone marrow-derived mast cell
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-determining region
CE-SDS	capillary electrophoresis sodium dodecyl sulfate
CH	Constant heavy
CL	Constant light
CMV	Cytomegalovirus
CTAs	Cancer testis antigens
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor

DC	Dendritic cell
EB	Evans Blue
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
FBP	Folate binding protein
Fc	Fragment crystallisable
FcR	Fc receptor
FGF	Fibroblast growth factor
FR α	Folate receptor alpha
FSC	Forward scatter
GPI	glycophosphatidylinositol
HC	Heavy chain
HDM	House dust mite
HER2	human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HRP	horseradish peroxidase
IC	Immune complex
i.c.	Intracellular
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation

i.p.	Intraperitoneal
i.v.	Intravenous
IFN γ	Interferon- γ
IMDM	Iscove's modified Dulbecco's medium
ITAM	Immunoreceptor tyrosine-based activation motifs
K _d	Dissociation constant
LC	Light chain
LT	Leukotriene
mAb	Monoclonal antibody
MAC	Membrane attack complex
MC	Mast cell
MCP-1	Monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cells
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NET	Neutrophil extracellular trap
NK	Natural killer
ns	Non-significant
OVA	Ovalbumin
PAF	Platelet-activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBST	PBS-tween
PD-L1	Programmed death-ligand 1

PLC	Phospholipase C
ROS	Reactive oxygen species
PCR	Polymerase chain reaction
s.c.	Subcutaneous
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SSC	Side scatter
SV40-Tag	SV40 large T antigen
TAM	Tumour-associated macrophages
TBI	Total body irradiation
TBST	Tris-buffered saline -tween
TCR	T cell receptor
T _{eff}	Effector T cell
TGF- β	Transforming growth factor-beta
Th	T helper
TIC	Thiophilic interaction chromatography
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TMB	3,3', 5,5;-tetramethylbenzidine
TME	Tumour microenvironment
TNF α	Tumour necrosis factor alpha
TRAMP	Transgenic adenocarcinoma mouse prostate
T _{reg}	Regulatory T cell

UPLC	Ultra Performance Liquid Chromatography
VEGF	Vascular endothelial growth factor
VH	Variable heavy
VL	Variable light
WB	Western blot
wt	Wild type

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Chapter 1: Introduction

This chapter aims to give a general overview of the use of monoclonal antibodies in cancer treatment, with a strong emphasis on IgE isotype. First, the readers will get familiar with antibody structure, the mechanism of action of tumour-targeting mAbs and the importance of correct antibody isotype selection. Then, a more in-depth review of current knowledge about IgE and cancer, also referred to as the AllergoOncology field, will be given. Finally, mast cells (MCs) and basophils - IgE effector cell types that are well-known in allergies but greatly overlooked in cancer – are discussed, as those cell populations were of particular interest in the work presented in this thesis.

1.1 Antibodies as cancer therapeutics

Monoclonal antibodies (mAbs) are among the fastest-growing class of drugs, with more than 100 mAbs with marketing approval since 1986¹. Most of them belong to cancer therapeutics², where their introduction has been a game-changer. Technological breakthroughs in antibody production made it possible to develop highly specific mAbs against a number of tumour antigens that could not be targeted before. Due to this unprecedented specificity and ability to selectively target the tumour cells, mAbs are often referred to as magic bullets, and their therapeutic application made it possible to cure previously untreatable cancers. However, the antibody Fab-arm, which dictates the antigen binding, is only half of the puzzle. It is the Fc tail and, therefore, the antibody isotype that significantly determines the downstream effector function and the subsequent activation of the immune system.

1.1.1 Antibody isotypes and their structure

Antibodies are antigen-specific immune molecules produced by B cells. They can be either anchored to the B cell membrane acting as a B cell receptor or secreted into the circulation and lymph by mature B cells, called plasma cells. Secreted antibodies provide humoral immunity by attacking and neutralising their corresponding antigens. There are five main isotypes of human antibodies – IgG (IgG1-IgG4), IgA (IgA1 and IgA2), IgM, IgE and IgD (**figure 1.1**). Although somewhat different structurally, they all share a standard structural organisation consisting of two heavy and two light chains joined by disulphide bridges and non-covalent bonds.

Heavy chains are composed of one variable domain (VH) and multiple constant domains (CHs), being the CHs identical for the antibodies of the same isotype/subclass. Light chains can be of either lambda or kappa isotype, and they consist of one variable domain (VL) and one constant domain (CL). From a more functional perspective, antibodies have two essential subunits: Fab (Fragment antigen binding) arm and Fc (Fragment crystallisable) tail. In IgG, IgD and IgA, Fab arm and Fc tail are connected by a stretch of amino acids called hinge region (**figure 1.1**). Fab arms mediate specific antigen binding dictated by hypervariable complementarity-determining regions (CDRs), with three CDRs present on each VH and VL domain. On the other hand, the Fc tail mediates antibody effector functions by binding to Fc receptors on immune cells and activating the complement system.

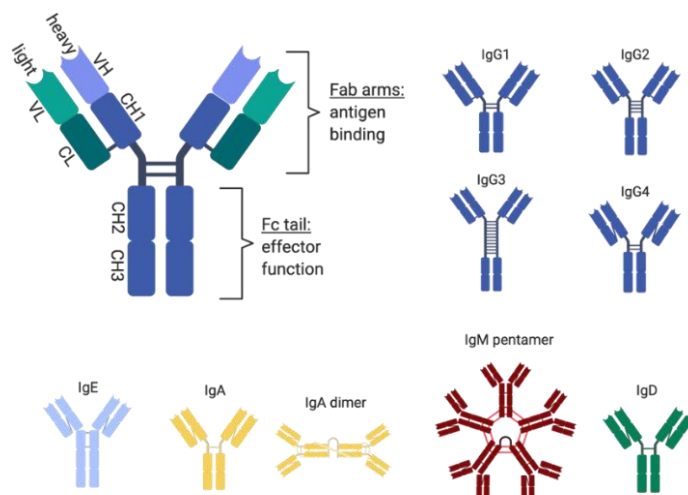


Figure 1.1. Antibody isotypes and structures. Structures of different antibody isotypes and subclasses are given. Although they share a standard structure, some particularities exist: (1) IgG3 is characterised by a very long hinge region; (2) IgE and IgM have one additional CH domain which replaces the hinge region; (3) IgM predominantly exists in the pentameric form; (4) Secretory IgA is mainly found as a dimer. Figure reprinted from Vukovic et al.³

1.1.2 Mechanism of action of tumour antigen-targeting mAbs

Most of the mAbs used in cancer therapy target tumour antigens which are, to varying extent, involved in tumour survival, growth and invasiveness. Interfering with tumour cell signalling pathways can induce tumour cell death on its own (e.g. anti-HER2, anti-EGFR)^{4,5}; however, it has been established that Fc-mediated activation of immune system substantially contributes to tumour cell destruction^{5,6}. With their Fc tail, antibodies can engage the complement system and different effector cells such as natural killer cells and macrophages, mediating in that way

antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) against tumour cells^{6,7}. These downstream antibody effects ultimately lead to tumour destruction. Furthermore, by mediating the tumour cell death and tumour antigen release, antibodies also induce the formation of immune complexes (IC), subsequent antigen cross-presentation and initiation of T cell response. Importantly, Fc binding to Fc receptors and complement activation are critical for cross-presenting tumour antigens by dendritic cells (DCs)^{8,9}. Schematic representation of different Fc downstream effects involved in tumour destruction is given in **figure 1.2**.

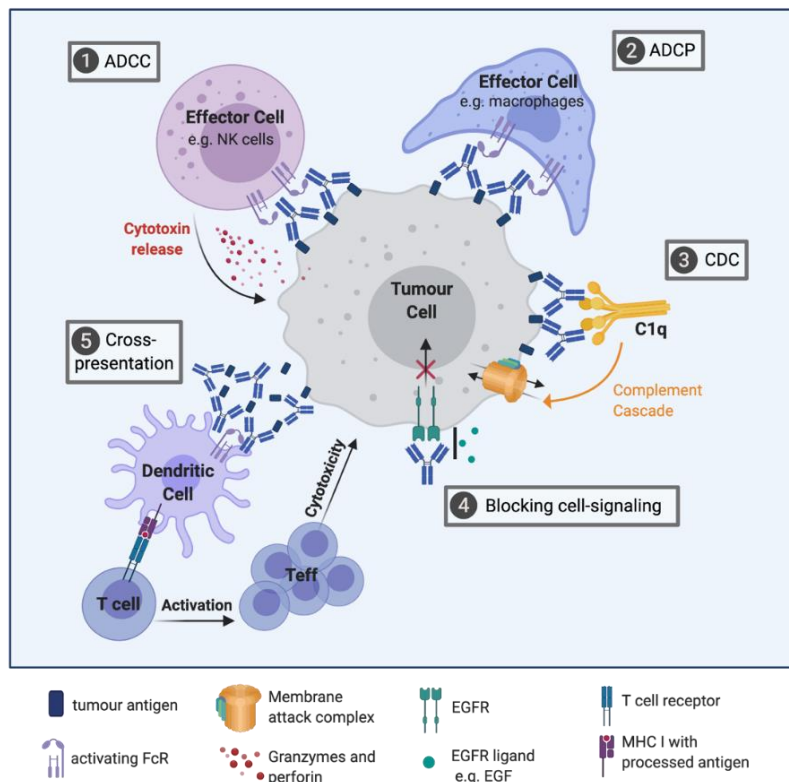


Figure 1.2. Mechanisms of action of tumour antigen-targeting antibodies. Antibodies mediate tumour cell death via multiple mechanisms: (1) ADCC, by inducing the release of cytotoxic mediators from the effector cell; (2) ADCP, by recruiting the effector cells for phagocytosis; (3) CDC, by initiating the complement cascade, which results in the formation of MAC; (4) blocking of essential cell-signalling pathways; (5) initiation of cross-presentation and adaptive immune response. Figure reprinted from Vukovic et al.³

1.1.3 Isotype selection for antibody-based cancer therapy

As already discussed, Fc-mediated effects play an important role in the antibody treatment outcome. Since different antibody isotypes bind to different FcRs on immune cells and differ in their potential to activate the complement system, they can induce diverse immune

responses. Therefore, antibody isotype should be carefully chosen during therapeutic mAbs development depending on the mechanism of action one wants to achieve with a specific antibody and the necessity for Fc-mediated effects. In the case of tumour-antigen targeting mAbs, the isotype with the highest capacity to induce ADCC, ADCP and CDC would be expected to be the most efficient. Although IgG1 has been traditionally used as the most active human isotype, IgE and IgA are currently being explored as promising alternatives. In addition, different Fc-engineering strategies have been developed to further optimise IgG effector function in terms of improving the activating-to-inhibitory (A/I) ratio and complement activation capacity. Detailed information regarding isotype selection for tumour antigen-targeting antibodies, checkpoint inhibitors and agonistic antibodies can be found in a review article that we published³. Here, I will discuss in more detail the current knowledge regarding the use of IgE antibodies as anti-tumour agents.

1.2 IgE biology

Together with MCs and basophils as their central effector cells, IgE antibodies are components of adaptive Th2 immune response. IgE class of antibodies is best known for mediating allergic reactions, including potentially lethal anaphylaxis. However, it has been well established that the physiological function of IgE is protection against parasites¹⁰ and animal venoms^{11,12}. What is more, it is believed that allergies have evolved from this potent protective role, like a misdirected response against a variety of primarily harmless antigens. The original role of IgE antibodies in fighting the tissue parasites has armed this antibody class with specific characteristics that can be exploited in cancer treatment. These are a direct consequence of the high-affinity binding of IgE to FcεRI, as will be discussed later.

1.2.1 Fcε receptors – structure, expression and IgE-mediated effects

Two IgE receptors have been described: the high-affinity FcεRI and the low-affinity FcεRII (CD23). FcεRI exists in either αβγ₂ tetrameric form or αγ₂ trimeric form (**figure 1.3**). Chain α is responsible for IgE binding, whereas β and γ chains mediate cell signalling. While the γ chain contains an ITAM sequence necessary for signal transduction, the β chain, although dispensable, helps the signal amplification and the FcεRI cell surface expression. The role of the β chain in expression amplification is reflected by the fact that tetrameric form is expressed at 10-100 fold higher levels than the trimeric one.

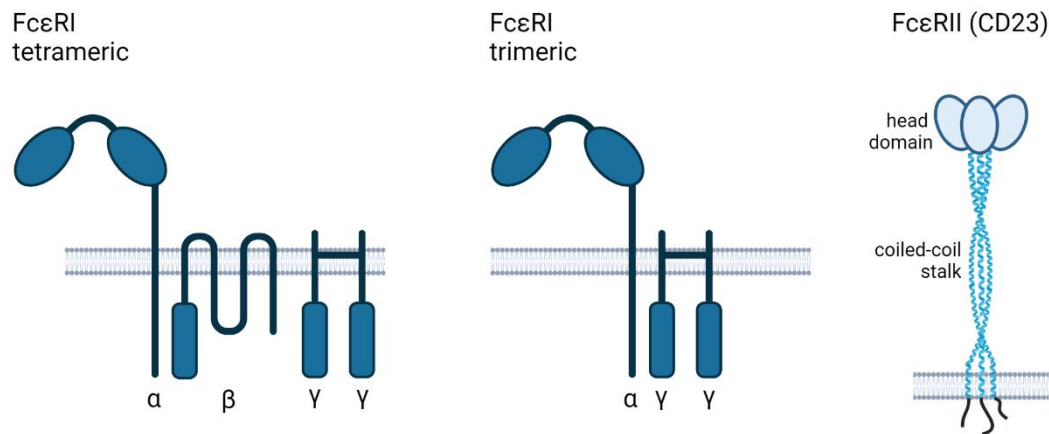


Figure 1.3. Schematic representation of FcεRs structure. FcεRI exists in either tetrameric (left) or trimeric form (middle). FcεRII belongs to the C-type lectin-like superfamily and it comprises C-terminal calcium-dependent (C-type) lectin-like domain ‘head’, followed by an α -helical coiled-coil stalk and a transmembrane domain (right).

Notably, the FcεRI expression profile shows significant inter-species differences (**table 1.1**)¹³. In contrast to humans, where both tetrameric and trimeric forms exist, mice only express the $\alpha\beta\gamma_2$ tetrameric form. Because the tetrameric form is expressed solely on MCs and basophils, these are the only two cell types known to express FcεRI in mice. In humans, on the other hand, FcεRI is also expressed as $\alpha\gamma_2$ trimer on monocytes, macrophages, dendritic cells, eosinophils, platelets and B cells (**table 1.1**). Thus, mice are not a suitable model for the complete study of IgE-mediated biological effects. On the contrary, rats show a similar FcεRI expression pattern to humans, with both tetrameric and trimeric forms present^{14–17}. Therefore, in terms of IgE biology, rats are physiologically closer to humans and represent a better animal model than mice.

The differences in the presence of FcεRI forms between mice and human can be explained by the expression regulation¹⁸. It has been demonstrated that co-expression of FcεRI γ -chain is necessary for FcεRI α -chain to reach the surface. Mechanistically, the γ -chain masks an ER-retention motif in the α -chain, allowing in such a way the export of a functional FcεRI complex¹⁹. Whereas the γ -chain presence is required for FcεRI cell-surface expression in both mice and human, the co-expression of β -chain is dispensable in humans but essential in mice. This suggests that additional ER-retention signals exist in murine FcεRI α -chain and are

masked by FcεRI β-chain. Therefore, in mice, FcεRI cannot be expressed on cell surface in the αγ2 trimeric form²⁰.

Table 1.1. Cellular expression profiles of Fcε receptors in human, mice and rats

	HUMAN			MICE		RAT		
Rc TYPE →	FcεRI αβγ2	FcεRI αγ2	CD23 (FcεRII)	FcεRI αβγ2	CD23 (FcεRII)	FcεRI αβγ2	FcεRI αγ2	CD23 (FcεRII)
CELL TYPE ↓								
MCs	x			x		x		
basophils	x			x		x		
monocytes		x	x				x	x
macrophages		x	x				x	x
DCs		x	x					
eosinophils		x	x				x	x
platelets		x	x					x
B cells		x	x		x			x
some T cells					x			

The binding of IgE to FcεRI and subsequent receptor crosslinking is the basis of allergic response^{21,22}. In the first ‘sensitisation’ phase, an individual is exposed to the allergen, after which B cell class-switching occurs under IL-4 stimulation and allergen-specific IgE antibodies are produced. The IgE then binds to FcεRI on MCs and stays bound for prolonged periods of time, making them ‘sensitised’ and ready for immediate reaction upon the next allergen exposure. Sensitised MCs initiate an allergic response in each subsequent allergen encounter, consisting of the acute and late phases. The acute phase is immediate and occurs within minutes after allergen exposure. It is dominated by MC degranulation, during which different mediators are released, such as histamine, proteases, IL-4, prostaglandins, leukotrienes and serotonin. In addition, the production of chemokines is induced. The symptoms in the acute phase are mostly histamine-related and, depending on the site of an allergic reaction, they can vary from bronchoconstriction in asthma to runny nose in allergic rhinitis and, most dramatically, anaphylactic shock if the allergen is encountered in the systemic circulation. The mediators released by MCs in the acute phase initiate the late phase within a few hours after allergen exposure. The late phase of allergic response is characterised by immune cell infiltration leading to a sustained inflammatory response dominated by eosinophils. Since IgE bound to FcεRI is extremely long-lived (see section 1.3.2), IgE direct involvement in late phase

allergic response cannot be excluded. Thus, IgE could mediate a sustained inflammatory reaction that could be exploited as anti-tumour mechanism.

The second IgE receptor is FcεRII, also known as CD23²³. CD23 is the only Ig receptor belonging to the C-type lectin-like superfamily, not the immunoglobulin superfamily. It comprises a C-terminal calcium-dependent (C-type) lectin-like domain 'head', followed by an α-helical coiled-coil stalk and a transmembrane domain (**figure 1.3**). The stalk region can be digested by endogenous proteases, leading to the release of soluble monomeric and trimeric forms of CD23, which upregulate IgE synthesis^{24,25}. Similarly to FcεRI, the CD23 expression profile also shows inter-species differences¹³ summarised in **table 1.1**. IgE binding to CD23 induces a variety of effects. For example, CD23 expressed on B cells is involved in IgE synthesis regulation. CD23 on intestinal epithelial cells mediates the transepithelial transport of the IgE immune complexes from the mucosal to the serosal side^{26–28}. Thus, CD23 is involved in the pathophysiology of food allergies by mediating the allergen transport from the entry site (intestinal lumen) to the underlying tissue where MCs are present. Furthermore, CD23 is involved in antigen presentation, as it internalises IgE-antigen complexes on antigen-presenting cells²⁵. Finally, it has been reported that IgE induces the ADCP effect via CD23 expressed on monocytes²⁹.

1.3 IgE and cancer

1.3.1 Epidemiological, preclinical and clinical evidence in the field of AllergoOncology

Several epidemiological studies have evaluated the role of IgE and allergy in cancer, with contradicting results. For example, an inverse correlation has been found between allergy/atopy incidence and certain types of cancer such as pancreatic, colorectal, glioma, childhood leukaemia and Hodgkin lymphoma^{13,30}. In addition, increased serum IgE levels seem to be correlated with an overall decreased malignancy risk and better prognosis³¹. Furthermore, tumour-specific IgE antibodies have been isolated from pancreatic cancer patients and have been shown to mediate ADCC against pancreatic cancer cells³². On the contrary, asthma and atopic dermatitis have been linked with an increased risk of lung cancer³³ and non-melanoma skin cancers³⁴, respectively. Thus, the evidence supports the existence of a complex relationship between IgE immune response and cancer. Recently, an

interesting combinatorial hypothesis has been published in an attempt to explain such a complex interplay³¹. The authors propose the combination of four different IgE/allergy-induced effects that can have a role in cancer development and immunosurveillance.

Firstly, chronic inflammation and tissue remodelling are common at the site of an allergic reaction, creating a pro-tumorigenic microenvironment. Secondly, allergy promotes type 2 immune skewing, moving the balance away from the Th1 inflammatory response important for tumour control. On the other hand, allergy is generally related to immune hyperresponsiveness and enhanced immunosurveillance. High levels of IgE antibodies can, therefore, mediate cancer immunosurveillance and activate a range of potent effector cells against tumour cells. Finally, allergy symptoms themselves, such as sneezing or coughing, can be beneficial as they help expel potential carcinogens and mutagenic compounds from the body. The overall result of these four types of effect is that allergic patients have increased cancer risk at the primary site of the allergic reaction due to chronic inflammation and Th2 skewing, but a lower incidence of cancer at distant sites due to enhanced IgE immunosurveillance and prophylaxis provided by allergy symptoms. This explanation fits with the epidemiological findings in asthma and atopic dermatitis. Both asthma and atopic dermatitis patients are at increased risk of cancer at the primary site of allergic manifestation – lung and skin, respectively. However, asthma and atopic dermatitis are protective against other types of cancer at distant sites such as colorectal, glioma, stomach and multiple myeloma³¹.

A similar dual connection of allergy/IgE and cancer exists in mice. For example, endogenous IgE antibodies have been reported to be protective in a mouse model of epithelial carcinogenesis³⁵. In this model, the topical application of a DNA-damaging agent DMBA induced a stress surveillance response in $\gamma\delta$ TCR+ intraepithelial lymphocytes and subsequent class switching in B cells to IgE isotype. Produced endogenous IgE antibodies protected the mice against carcinogenesis. This anti-tumorigenic role of IgE was confirmed by lack of protection in *Igh7^{-/-}* and *Fcer1a^{-/-}* mice deficient for IgE heavy chain and Fc ϵ RI, respectively. Similarly, endogenous IgE antibodies have been reported to exert immunosurveillance against transplanted tumours^{36,37}. For instance, Nigro et al. demonstrated that both IgE knockout mice and Fc ϵ RI knockout mice were more susceptible to the growth of transplantable tumours when compared to wild type mice. On the contrary, the tumour

growth was significantly reduced in KN1 mice, which are characterised by an increased number of IgE-secreting B cells and IgE serum titer. Observed tumour protection was lost when CD8⁺ T cells were depleted, indicating that IgE-T cell cross-talk was crucial for tumour control.

In contrast to this protective role of IgE antibodies in carcinogenesis, it has also been reported that inflammation-induced IgE promote epithelial tumour growth³⁸, further supporting the chronic inflammation hypothesis. The authors showed that skin inflammation led to increased IgE levels and subsequent basophil recruitment to the skin via CXCL12 and CXCR4. IgE-activated basophils in the inflamed skin then promoted the tumour growth, partly via histamine. Thus, the findings in mice indicate that IgE antibodies play a role in tumour immunosurveillance, but chronic inflammation may subvert their protective role towards a pro-tumorigenic function.

Overall, the epidemiological, preclinical and clinical data highlight the complex relationship between allergy and cancer where both pro-tumorigenic and anti-tumorigenic effects are possible, depending on the context. If used wisely, these insights could guide and shape the future use of IgE antibodies in cancer, for example, in terms of identifying the types of cancer or the patient groups most likely to benefit from IgE treatment.

1.3.2 Unique IgE properties and its comparison to IgG

IgE offers a few advantages over IgG that can be exploited in cancer therapy. For instance, the affinity of IgE for its central receptor FcεRI ($K_d=10^{-10}$) is two orders of magnitude higher than the one of IgG for its high-affinity receptor FcγRI ($K_d=10^{-10}$ vs $K_d=10^{-8}$, respectively)¹³. Additionally, IgE shows a prolonged dissociation rate from FcεRI and restricted tissue diffusion²². Consequently, IgE antibodies stay bound to FcεRI-expressing cells for a prolonged time, even in the antigen absence. Thus, FcεRI⁺ cells are constantly ready for an immediate response upon antigen exposure, making the FcεRI an anticipatory receptor with potential immunosurveillance properties. Since FcεRI-expressing cells are mostly tissue-resident, IgE has an extended tissue half-life of around two weeks^{39,40}. On the contrary, the serum half-life of IgE is very short, and it reaches only 2-3 days⁴¹. It is suggested that this characteristic is directly connected to the original role of IgE in tissue immunity anti-parasitic response. For IgG, the opposite is true: it has a tissue half-life of 2-3 days and a serum half-life of 2-3 weeks⁴².

Not surprisingly, IgG monoclonal antibodies have shown much greater efficacy in treating haematological cancers than solid ones. Thus, the extended tissue half-life makes the IgE isotype a much better candidate for solid tumour targeting.

Furthermore, due to very low endogenous levels of IgE in the blood, exogenously administered IgE treatment would encounter minimal competition for receptor binding. If we look at the immunoglobulin composition in the systemic circulation, 85% are IgG, and only 0.02% are IgE antibodies⁴³. In fact, it has been shown that high endogenous levels of IgG translate into the loss of IgG efficacy and the need for higher doses⁴⁴. Thus, low systemic endogenous competition combined with prolonged tissue half-life indicates that therapeutic IgE antibodies would require lower doses and less frequent administration. Finally, another advantage of IgE isotype is that there are no known inhibitory Fc receptors for IgE, as opposed to FcγRIIb for IgG, which causes immunosuppression¹³. Summary of main advantages of IgE isotype over IgG is given in **table 1.2**.

On the other hand, short serum half-life and low circulating concentrations of IgE isotype may pose a disadvantage in the treatment of haematological cancers. Thus, it is tempting to speculate that IgG plasma surveillance and IgE tissue surveillance may be considered as complementary mechanisms in fighting the cancer.

Table 1.2. IgE and IgG comparison

	IgE	IgG
Receptor affinity	Kd=10 ⁻¹⁰	Kd=10 ⁻⁸
Anticipatory receptor	Yes	No
Serum t1/2	2-3 days	2-3 weeks
Tissue t1/2	2 weeks	2-3 days
Endogenous competition	+	+++
Inhibitory receptor	No	Yes

1.3.3 The use of monoclonal IgE antibodies as cancer therapeutics

As discussed above, several epidemiological and clinical observations suggested a potential tumour-protective effect of allergies and IgE antibodies. In addition, the IgE isotype shows

multiple advantages compared to IgG. Taken together, there is a strong rationale for exploring the potential use of mAbs of the IgE isotype as anti-tumour agents.

Preclinical data

The first study that laid the cornerstone for IgE mAbs in cancer was done in 1999 by Gould et al.⁴⁵ The authors directly compared the tumour-killing capacity of IgE and IgG1 targeting folate receptor alpha (FR α) in SCID mice. Here, a xenograft model was used with subcutaneous transplantation of IGROV1 human ovarian cancer cell line expressing folate binding protein (FBP). In combination with human PBMC provided as effector cells, IgE exhibited significantly longer-lasting protection against the tumour growth than IgG1 plus PBMC: 35 days versus 19 days, respectively. This anti-FR α IgE antibody, also known as MOv18, has since then been extensively studied both *in vitro* and *in vivo*, shaping our current understanding of anti-tumour mechanisms of IgE isotype.

Of note, due to already mentioned inter-species differences in Fc ϵ RI cellular distribution, the creation of human Fc ϵ RI α transgenic mouse has been of great importance for studying the human IgE biology^{46–48}. In these mice, murine Fc ϵ RI α (IgE-binding subunit) was replaced with human Fc ϵ RI α . The immunoprecipitation experiments showed that the human α subunit formed complexes with both β and γ murine subunits, making a chimeric receptor. Surprisingly, the replacement of the α subunit also changed the expression profile of Fc ϵ RI. With humanised Fc ϵ RI being expressed on monocytes, eosinophils, Langerhans cells and DC, in addition to MCs and basophils, this transgenic mouse mimics the human repertoire of IgE effector cells, making it a much more accurate IgE model. Of note, these mice are not transgenic for human Fc ϵ RII. Thus, the effect that human IgE may mediate via Fc ϵ RII cannot be studied in this model. In addition to human Fc ϵ RI α transgenic mice, rats are also occasionally used as an immunocompetent model.

Several studies established myeloid cells as the central IgE anti-tumour effector population^{15,49}. Further *in vitro* characterisation showed that IgE-mediated monocyte activation could result in both ADCC through Fc ϵ RI, as well as ADCP through Fc ϵ RII²⁹. A breakthrough study regarding the IgE-myeloid cells axis in tumour context was published in 2017 by Josephs et al.¹⁵ Here, a syngeneic rat model was used to compare the efficacy of Mov18 rat IgE and rat IgG2b (equivalent to human IgG1) in preventing lung metastases

formation following intravenous injection of CC531tFR rat colon adenocarcinoma cells. Not only was IgE superior to IgG in controlling the tumour growth, but it also induced increased intra-tumoral infiltration by macrophages. Remarkably, intra-tumoral macrophages from IgE treated rats were differentiated towards activated M1-like phenotype characterised by upregulation of a TNF α /MCP-1/IL-10 cytokine signature. Therefore, in addition to recruiting tumour-associated macrophages (TAM) for direct tumour killing via ADCC and ADCP, IgE antibody also re-educated them towards anti-tumour M1 phenotype, reshaping the tumour microenvironment (TME) in a beneficial way⁵⁰.

Another mechanism shown to contribute to IgE anti-tumour response is enhanced antigen cross-presentation. Several approaches were used, such as the vaccination with the *ex vivo* IgE-antigen loaded DCs⁵¹ or the IgE preincubated with tumour antigen⁵². These studies have established that IgE-mediated cross-presentation participates in the anti-tumour effect of IgE antibodies *in vivo* by initiating a T cell response against tumour cells.

In addition, it has been shown that trastuzumab IgE can reduce cell viability of HER2⁺ human breast adenocarcinoma cell line without effector cell presence to similar extent as trastuzumab IgG⁵³. This finding confirms that IgE antibodies can induce tumour cell death via Fab-mediated blocking of cell growth receptors and independently of Fc ϵ R downstream effects. Thus, similarly to IgG, both Fab-mediated and Fc-mediated effects of IgE play a role in tumour cell killing and act synergically.

Clinical data

Following the extensive *in vitro* and *in vivo* characterisation of MOv18 IgE, this antibody has entered the phase I clinical trial as the first IgE-based cancer therapy (NCT02546921). Twenty-four patients with solid FR α + tumours have been recruited, and the first findings recently published⁵⁴. Although very low total IgE doses were used (0.07-3.0mg), preliminary evidence of anti-tumour activity has been achieved in one patient at a total IgE dose of only 0.7mg, represented by the shrinkage of peritoneal metastases and tumour marker reduction. Anti-tumour IgE efficacy in human settings remains to be evaluated in more extensive phase 3 clinical studies, where probably higher doses will be used. Nevertheless, the MOv18 IgE phase I results are promising, and, thus, further clinical testing is warranted.

Safety concerns

Probably the most significant concern regarding the therapeutical use of IgE mAbs is the risk of anaphylaxis, which could be life-threatening if degranulation of MCs or basophils is induced in the systemic circulation. Theoretically, tumour antigens are released into the blood as monomers and, therefore, would not be sufficient to induce the crosslinking of FcεR-bound IgE required for degranulation⁵⁵. On the other hand, circulating tumour cells express multiple copies of the target antigen and could potentially induce IgE-mediated degranulation. Consequently, the risk of anaphylaxis has been extensively evaluated in preclinical models. However, the safety data was reassuring^{55–57}, with the absence of anaphylactic symptoms and signs in IgE-treated animals. The satisfactory preclinical safety gave a green light for initiating the phase I clinical trial mentioned above⁵⁴. Patients were evaluated for the potential systemic allergic reactions by pre-treatment skin prick test and ex vivo basophil activation test (BAT). Urticaria has been reported as the most common side effect. Only one patient suffered from an anaphylactic reaction, and, interestingly, that was the only patient with a positive pre-treatment BAT test. Thus, the positive BAT test is now used as the exclusion criteria. Taken together, the safety of IgE mAbs seems acceptable, the side-effects manageable, and the BAT test has been identified as an essential tool to minimise the anaphylaxis risk.

1.4 Mast cells and basophils as potential IgE effector cell population in cancer treatment

The vast majority of preclinical studies in the field of AllergoOncology have focused on IgE antibodies against surface antigens and subsequent activation of macrophages or dendritic cells. However, it seems surprising that the best known IgE effector populations – MCs and basophils – have been overlooked. In addition, in the allergic response, IgE binds to soluble and not surface antigens. Therefore, MCs and basophils may be significant additional IgE effector cell populations in the fight against cancer. For that, we need to discuss the current understanding of the role of these immune cells in cancer.

1.4.1 Mast cells and cancer

The presence of MCs in tumours was described by Paul Ehrlich in his doctoral thesis already in 1878⁵⁸. Today, it is well-established that MCs are among the first immune cells to infiltrate the tumour microenvironment⁵⁹. Increased MC density in TME has been reported for several

solid and hematologic cancers^{60–65}. However, the precise effects of MCs on tumour growth remain controversial⁶⁶, since the presence of MCs in tumours has been associated with both unfavourable and favourable prognosis^{67–72}. In fact, the growing body of evidence suggests that the role of MCs in cancer depends on the tumour type and stage, as well as MC microlocalisation within the tumour⁶⁵ and MC phenotype⁷³. As it has been recently reviewed by Varricchi et al.⁶⁵, there are certain types of cancer in which MCs have been reported to be pro-tumorigenic, others in which MCs are anti-tumorigenic, and a few in which both pro-tumorigenic and anti-tumorigenic roles have been described (**figure 1.4**).

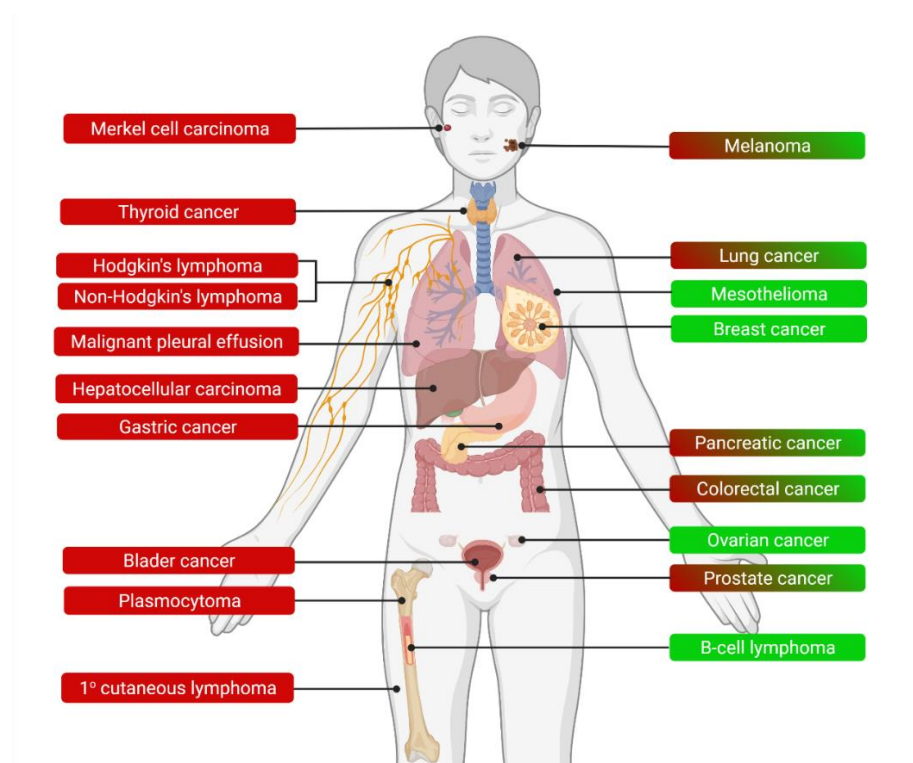


Figure 1.4. MCs in human tumours. Types of cancer in which MCs play a pro-tumorigenic role are given in red boxes. The ones in which MCs appear to be protective are given in green boxes. The tumours in which MCs have been reported to play both pro-and anti-tumorigenic roles are shown in red/green boxes. Figure adapted from Varricchi et al.⁶⁵

In addition, the data suggest that the role of MCs in carcinogenesis can be different in the early and late phases of tumour development^{65,74}. Furthermore, it has been established that intratumoral and peritumoral MCs can also lead to opposite outcomes during carcinogenesis^{65,75}.

The complex role of MCs in tumorigenesis is not surprising, as they can interact not only with tumour cells but also with other immune cell populations and extracellular matrix. The most

important MC-derived mediators involved in carcinogenesis are summarised in **figure 1.5**. On the one hand, tumours attract MCs from very early time points of development in order to establish sufficient vascularisation to support tumour growth⁷⁶. Due to this angiogenic and lymphangiogenic effect, mediated mainly by the members of the VEGF family, MCs have traditionally been seen as pro-tumorigenic. The pro-tumorigenic role is further supported by immunosuppression induced by MC-derived TGF- β , IL-10 and adenosine⁷⁷.

On the other hand, however, MCs could also have a direct cytotoxic effect, as suggested by histological images of degranulating MCs surrounding dying tumour cells in breast cancer⁷⁸. The release of cytotoxic compounds from MCs, including tumour necrosis factor- α (TNF α), leads to inflammation, cytotoxic T lymphocyte (CTL) responses, and tumour destruction¹³. For instance, the presence of TNF α -releasing MCs and higher degranulation index may be correlated with better survival of non-small cell lung cancer patients⁷⁹. Furthermore, MCs can mediate anti-tumour effects indirectly through the recruitment of the immune cells and the enhancement of their anti-tumour activity⁸⁰. MCs recruit the immune effector cells via a complex process that includes the release of a variety of chemokines, such as CCL3, CCL5, CXCL10, CXCL8 and LTB₄, increase in the vascular permeability (most notably via histamine) and up-regulation of adhesion molecules on endothelial cells through IL-1 β and TNF⁸⁰. In addition, MCs have been reported to enhance the activity of NK cells and effector T cells. For instance, MCs are an important source of IL-6^{81,82} that increases IFN- γ positive tumour-infiltrating lymphocytes, subsequently leading to tumour growth reduction *in vivo*⁸³. Next, activated MCs also stimulate NK cells to secrete IFN γ ⁸⁴, one of the central players in anti-tumour response. Indeed, a recent study in an immunosuppressed preclinical model of pancreatic cancer demonstrated that the addition of IgE treatment to the anti-PD-L1 and TLR3 agonist induced a robust, NK and CD8⁺ T-cell mediated anti-tumour response⁸⁵. The tumours from the treated mice had a decreased percentage of TIGIT⁺ PD-1⁺ NK cells. Both TIGIT and PD-1 are well-known checkpoint inhibitors that suppress the activity of NK cells. In addition, the NK cells from the mice treated with the triple combination therapy showed an increased degranulation *ex vivo*, further confirming their enhanced cytotoxic activity.

Taken together, the data suggest an ambiguous role of MCs in cancer, and they have been recognised as a promising candidate for cancer immunotherapy⁵⁹. However, MC targeting in

cancer could comprise two different approaches – promoting their cytotoxic activity on the one hand but inhibiting the angiogenic and immunosuppressive functions on the other. For a more in-depth overview of the role of MCs in the tumour microenvironment, the readers can consult excellent review articles published in recent years^{65,80,77}.

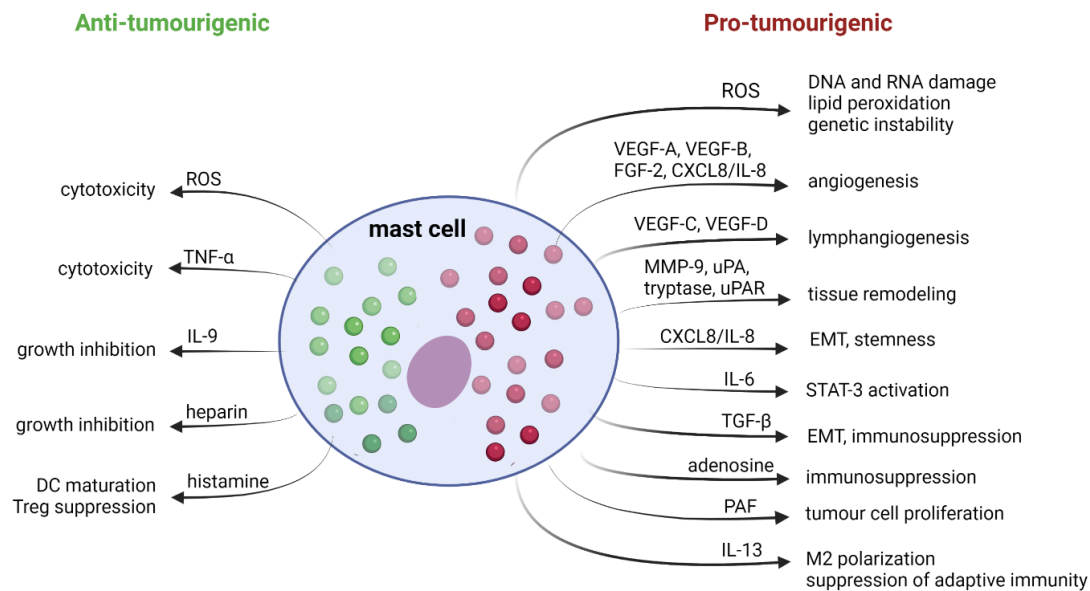


Figure 1.5. MC mediators that are involved in carcinogenesis and the effects they induce. On the one hand, MCs can have anti-tumour effects either by direct cytotoxicity mediated by TNF and ROS or indirectly - by growth inhibition (IL-9, heparin), and DC maturation and T_{reg} suppression (histamine). On the other hand, MCs promote tumour growth and metastasis by releasing angiogenic and lymphangiogenic factors, inducing epithelial-to-mesenchymal transition (EMT) and stemness, and mediating immunosuppression, M2 polarisation and tissue remodelling. Figure adapted from Varricchi et al.⁶⁵

1.4.2 T_{reg} and mast cell interplay

T regulatory cells (T_{regs}) critically contribute to the establishment of an immunosuppressive environment within tumours, and their therapeutic targeting can significantly enhance the efficacy of tumour treatments⁸⁶. It has been demonstrated that MCs are an “essential intermediate” in local tolerance induction by T_{regs}⁸⁷. Huang et al. reported that MC infiltration into solid tumours exacerbated local immunosuppression by increasing T_{regs} numbers⁸⁸. At the same time, our group showed that the MC-derived EGF-like growth factor, Amphiregulin, is essential for T_{regs} to establish an immune-suppressive tumour-intrinsic environment⁸⁹. MCs not only can enhance T_{reg} function but, on the contrary, MC degranulation triggered by IgE can also transiently diminish T_{reg} function, as shown by de Vries et al. in a skin allograft

tolerance model⁹⁰. When the authors induced MC degranulation, either systemically or intragraft, they observed the T-cell-mediated rejection of previously established, tolerant skin grafts. Mechanistically, MC degranulation led to transiently diminished T_{reg} function characterised by both a lower number of T_{regs} in the graft as well as downregulation of suppressor molecules including TGFβ, IL10 and granzyme B. In another study, MC-derived and exogenous histamine have been shown to inhibit T_{reg} suppressor function via H1 receptor⁹¹. Thus, MC degranulation induces rapid loss of T_{reg} function through histamine receptor 1, leading to acute T-cell inflammation. These findings directly correlate to the findings in the field of food allergy research, where it has become apparent that the presence of allergen-specific IgE substantially impairs T_{reg} development and function^{92,93}. If applied to the field of cancer, induction of MC degranulation could, therefore, be beneficial in breaking the T_{reg}-mediated immunosuppression and changing the cellular composition of TME in favour of CD8⁺ effector cells.

Interestingly, as early as 1977, Lynch & Salomon demonstrated that experimentally induced MC degranulation specifically within tumours induces tumour regression⁹⁴. The authors infused IgE antibodies into tumour-bearing mice and induced “passive local anaphylaxis” by injecting the antigen/allergen directly into the tumours. Such treatment resulted in complete regression of a significant number of tumours. Since the therapeutic effect was entirely reversible by the administration of histamine inhibitors, these data strongly suggest that tumour-intrinsic MC degranulation was the underlying mechanism that led to tumour rejection. This MC-mediated tumour rejection that Lynch & Salomon described could be possibly explained by histamine-mediated T_{reg} suppression. Thus, IgE-mediated MC activation seems promising, yet not fully explored, anti-tumour mechanism of IgE mAbs.

1.4.3 Basophils – biology and connection to cancer

Basophils represent a small percentage (<1%) of human peripheral leukocytes. Due to their similarities with MCs, such as the expression of the tetrameric form of FcεRI, similar granular content and their role in allergic reactions, they have long been described as a MC circulating analogue. However, this view has been abandoned due to the growing evidence that MCs and basophils have different morphology and ultrastructure, as well as the biochemical, pharmacological and immunological properties^{95–97}. In addition, although the general

understanding is that basophils are primarily present in the circulation and can only be found in the tissue under inflammatory conditions⁹⁸, this concept has recently been challenged by a study from Cohen et al., who described the presence of basophils throughout all phases of lung development⁹⁹. The identified lung basophils had a distinct phenotype to the peripheral blood basophils and were essential for the development of alveolar macrophages and their M2 polarisation.

The role of basophils in carcinogenesis is still poorly understood, although the limited data suggest a dual role, similarly to MCs. For instance, low basophil counts have been associated with a worse prognosis in colorectal cancer patients^{100,101}. Similarly, high basophil levels have been correlated with improved survival in ovarian cancer¹⁰² and a better outcome of melanoma patients treated with immune checkpoint inhibitors¹⁰³. Furthermore, a protective role of basophils has been reported in a mouse model of breast cancer¹⁰⁴. On the contrary, basophils seem to have a detrimental role in pancreatic¹⁰⁵ and bladder cancer¹⁰⁶.

1.4.4 Mechanisms of pro- and anti-tumorigenic roles of basophils

Similarly to MCs, basophils release several different mediators, which can have both detrimental and favourable effects during carcinogenesis. For instance, basophils represent a major source of multiple angiogenic factors, including VEGF-A and VEGF-B, HGF, ANGPT1 and CXCL8⁹⁸. In addition, basophils produce LTC4 and LTD4 that have been shown to promote angiogenesis via activation of CysLT2R expressed on endothelial cells¹⁰⁷. Furthermore, they can release basophil extracellular DNA traps (BETs)^{108,109}, similar to those released by neutrophils (NETs). The role of NETs in tumour growth and metastasis has been well established; thus, BETs would have similar effects, although studies are needed to confirm this hypothesis. Next, it is tempting to extrapolate the basophil involvement in M2 polarisation observed in lung development to cancer. In fact, it has been reported that basophils promote Th2 and M2 polarisation in a mouse model of pancreatic cancer via the production of IL-4¹⁰⁵, providing further evidence of their pro-tumorigenic role. Basophils were also a key IgE cell mediator in the chronic skin inflammation model, leading to tumour outgrowth³⁸, as discussed in section 1.4.1.

On the other hand, in a melanoma mouse model, following T_{reg} depletion, basophils induced CD8⁺ T cell infiltration into the tumour via CCL3 and CCL4¹¹⁰, ultimately resulting in melanoma

rejection. In addition, IL-33-activated basophils inhibited the growth of B16-F10 cells in co-culture¹¹¹. Since such an effect has not been observed with resting basophils, it appears that appropriate stimulation is needed for basophils to exert their tumouricidal effect. In support of this hypothesis, gene signatures of activated intratumoral basophils were associated with improved survival of ovarian cancer patients¹⁰². Finally, basophils represent a significant source of TNF α and histamine, whose anti-tumorigenic roles I already discussed in sections 1.5.1 and 1.5.2.

In summary, although less studied than MCs, it is becoming evident that basophils are involved in carcinogenesis in a similar way, with both protective and detrimental roles. Importantly, activated basophils seem to play an anti-tumorigenic role and thus, their activation by IgE antibodies is of interest for further studies.

1.5 Aims of the thesis

Given the increasing evidence suggesting the potential superiority of the tumour-antigen targeting antibodies of IgE isotype over IgG, the main aim of this thesis was to characterise anti-tumorigenic IgE capacity further. My work focuses on MCs and basophils as IgE effector cells, as these cell types have been minimally explored in the field of AllergoOncology. Importantly, I have studied the different outcomes of IgE-mediated activation of MCs and basophils depending on the type of antigen – surface, secreted or intracellular. This was done to mimic different antigen exposure types and consequent acute or chronic effector cell activation. Of note, all the *in vivo* work was done in wild type mice as a clean model system in which FcεRI is expressed solely on these two cell populations. However, the lack of FcεRI expression on other immune cells in mice may pose a limitation, as complex interactions with those immune cell populations upon IgE activation are lost.

Firstly, I developed an IgE purification method, as standard methods used for IgG purification, such as protein A or protein G chromatography, cannot be applied to IgE. For this purpose, I optimised the thiophilic interaction chromatography for IgE purification. This work has been published and is provided in the appendix section at the end of this thesis.

In Chapter 2, I focus on a **surface tumour antigen**. To this end, an anti-Thy1.1 antibody was used to target subcutaneously transplanted B16-OVA-Thy1.1 cells. Here, I directly compared the efficacy of the same tumour targeting antibody with different isotypes (IgG1, IgG2a and IgE), as IgG antibodies are also mainly used to target surface antigens. The results show that the anti-Thy1.1 antibody with an IgG2a isotype was superior to the IgG1 and the IgE isotype antibodies in controlling tumour growth in this setup. The absence of tumour growth control by IgE isotype suggests that MCs and basophils were probably poorly infiltrated in the TME and not in the close proximity of antigen-expressing cells. Thus, surface tumour antigens are probably not an ideal target for IgE-mediated MC/basophil activation.

Next, **In Chapter 3**, I wanted to target a **secreted antigen** as a way to model the chronic exposure to a soluble antigen. In addition, the effects of IgE in combination with OT-1 adoptive cell transfer were studied. Here, I combined two IgE antibodies targeting the same antigen (OVA) in order to achieve effector cell degranulation. My data show that IgE-mediated effects during chronic exposure to a soluble antigen abolish the protective effect of OT-1 cells.

This suggests that MCs and basophils may contribute to chronic IgE-mediated inflammation at the tumour site by creating an immunosuppressive TME. Thus, these findings further support the chronic inflammation hypothesis, according to which patients with allergies are at greater risk to develop cancer at the site where allergy is primarily manifested.

Furthermore, **in Chapter 4**, due to the lack of mAbs to target an intracellular tumour antigen, I took a different approach. Here, I immunised mice against an **intracellular antigen** under type-2 immune skewing and studied how the polyclonal immune response raised in such a way affects tumour development. This was done in both B16-OVA syngeneic model and spontaneous prostate tumour model (TRAMP mice). Although the data are not entirely conclusive, there are indications that type-2 immune skewing in combination with T cell adoptive cell transfer has protective effects. These findings, therefore, suggest that acute, short exposures to soluble antigens may be an optimal way to activate MCs and basophils against tumour cells.

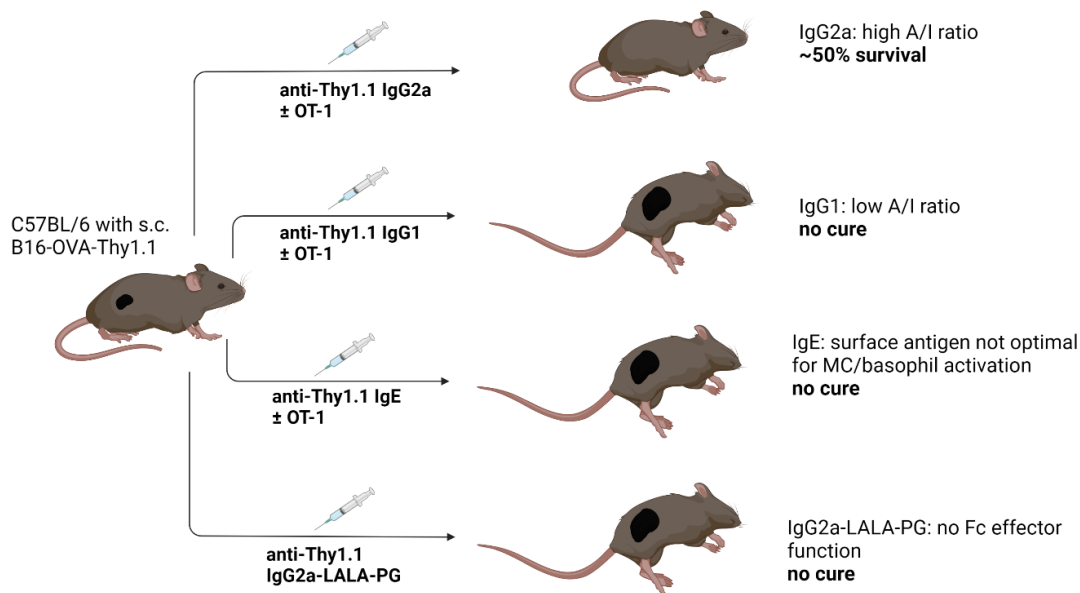
Finally, I developed human IgE bispecific antibodies as a novel antibody format that would combine the increased selectivity of bispecific antibodies with the potent effector mechanism of IgE isotype. This manuscript has been published, and its copy is provided in the thesis appendix. In short, I used two different strategies for IgE bispecific formation: knobs-into-holes and leucine zipper mediated molecule pairing. Both approaches resulted in fully functional bispecific IgE molecules. My results show that the bispecific IgE preserved the binding to both antigens and that the Fc functionality was not affected, as it successfully induced *in vitro* MC degranulation and *in vitro* and *in vivo* tumour killing. Importantly, bispecific IgE molecules were superior to the bispecific IgG1 homologue in ADCC-mediated *in vitro* killing of tumour cells when monocytes were provided as effector cells.

Overall, the presented work contributes to our current understanding of IgE-mediated effects on tumour development. It argues that IgE-based immunotherapies have different outcomes depending on the localisation of the targeted antigen and context. The beneficial effect was achieved only when intracellular antigens were targeted, indicating that might be an optimal way for IgE to activate the anti-tumour mechanisms of MCs and basophils. In addition, I have developed a couple of valuable tools for further IgE research – a universal and gentle IgE purification method and the first human IgE bispecific formats.

2 Chapter 2: IgG2a targeting a surface tumour antigen is superior to IgG1 and IgE in controlling the tumour growth in wild type mice

2.1 Abstract

In this chapter, I compared the efficacy of IgG2a, IgG1 and IgE against a surface tumour antigen (Thy1.1) in the B16-OVA-Thy1.1 model. My results show that only IgG2a successfully controlled tumour growth. The observed anti-tumour effect of IgG2a was Fc-mediated, as confirmed by loss of efficacy when silencing LALA-PG mutations were introduced in IgG2a Fc tail. The results also indicate that a cell surface antigen is not an optimal target for IgE-mediated MC and basophil activation in tumour settings. The combination of the tested antibody isotypes with OT-1 adoptive cell transfer did not make any difference in the tumour rejection. Thus, no synergistic effect was observed. The graphical abstract is given below.



C57BL/6 mice were subcutaneously injected with B16-OVA-Thy1.1 cells and treated with different anti-Thy1.1 antibody isotypes with or without OT-1 adoptive cell transfer. Positive outcome was only achieved in mice treated with anti-Thy1.1 IgG2a. The protective effect was lost when silenced IgG2a with abolished FcR binding was used (IgG2a-LALA-PG). Addition of OT-1 treatment did not make a difference in the treatment outcome.

2.2 Introduction

The introduction of monoclonal antibodies to cancer treatment critically contributed to better outcomes and increased survival for different types of cancer. However, many patients are still unresponsive to such tumour-targeting antibody therapy. This underlines the need for further optimisation of antibody-based approaches, in which isotype switching from IgG to IgE has shown promising results.

In preclinical models, IgE exhibited superior tumour control to its IgG homologs, and IgE mediated the anti-tumour effect mainly through macrophages and dendritic cells^{50,52}. However, due to critical differences in FcεR cellular expression between mice and humans, IgE mAbs were evaluated either in immune-deficient mice in which human cells were provided as effectors or in transgenic hFcεRIα mice⁴⁸. Therefore, the studies in wild type mice with the native immune system are lacking. In addition, the role of potent IgE effector cells, such as MCs and basophils, have not been addressed yet. Since FcεRI expression in mice is restricted to these two cell types, mice represent an appropriate model to study the outcome of IgE-mediated MC and basophil activation in cancer.

In this chapter, I addressed that question in a syngeneic mouse model where IgE monoclonal antibody against a surface tumour antigen was compared to IgG2a, as the most active IgG isotype in mice, and IgG1, as the least active. To this end, B16-OVA cells stably transfected to express Thy1.1 were used. Thy1.1 was chosen as a model tumour antigen for this study for a few reasons. Firstly, wild type mice express only Thy1.2. Thus, Thy1.1 would exclusively be present on tumour cells, making our treatment tumour-selective. Secondly, Thy1.1 would not be functionally important for the growth of the tumour cells in which it would be expressed, i.e. it would only serve as a binding site for the anti-Thy1.1 antibodies without any intracellular signalling interference. Therefore, the anti-tumour effect we would observe would solely be due to Fc-mediated effects, making it ideal for comparing different antibody isotypes. Finally, we had the OX7 hybridoma secreting anti-Thy1.1 antibodies available, giving us the chance to produce them in house.

In addition, the antibody treatment was combined with the adoptive transfer of activated OT-1 cells. OT-1 cells are CD8⁺ T cells that recognise the OVA peptide (SIINFEKL) in the context of MHC class I¹¹². Thus, in the case of the B16-OVA model, OT-1 cells can be used as tumour-

specific CD8⁺ T cells. The rationale for this combination treatment was the evaluation of potentially induced changes in the immune-suppressive tumour microenvironment. If any of the tested antibody isotypes attenuates such an unfavourable TME, OT-1 treatment could exert its full potential.

My results show that IgG2a was superior to both IgE and IgG1 in controlling tumour growth in this model. We confirmed that the observed anti-tumour effect was Fc-mediated as the protection was lost with IgG2a featuring silencing LALA-PG mutations. Furthermore, none of the tested antibody isotypes could rescue the efficacy of adoptively transferred OT-1 cells, indicating that the immune-suppressive tumour microenvironment in the B16-OVA model was not significantly changed with the treatment. Overall, these results indicate that a surface tumour antigen is not the ideal choice for IgE-induced MC or basophil activation in tumour settings. In addition, although Thy1.1 has proven to be difficult to express on B16-OVA cells, we managed to successfully express it by replacing its GPI anchor with the MHC-1 transmembrane domain. This might have a useful application in detecting transfectants.

2.3 Materials and methods

2.3.1 Antibody design, production and purification

Amino acid sequences of all anti-Thy1.1 antibodies are provided in **supplementary table 1**. The design and production of murine anti-Thy1.1 IgG1 and IgE is described in our published paper provided in thesis appendix¹¹³. The IgG2a HC and the IgG2a HC featuring silencing LALA-PG mutations were cloned using standard cloning techniques from plasmids available in house (anti-Siglec and anti-TNFR2, respectively) into the pcDNA3.1(+) encoding for anti-Thy1.1_VH (**Fig. 2.1**). Correct clones were confirmed by Sanger sequencing. The plasmid encoding for the anti-Thy1.1 light chain was *de novo* synthesised (GeneArt).

Anti-Thy1.1 IgG2a and anti-Thy1.1 IgG2a-LALA-PG were produced in ExpiCHO-S™ cells and FreeStyle293 cells, respectively, as described before¹¹³. Purification was done with MabSelect SuRe LX resin. Anti-Thy1.1 IgG2a had to be polished with preparative SEC (data not shown). Preparative SEC and the quality control consisting of UPLC-SEC, CE-SDS and SDS-PAGE were performed as described in our published paper¹¹³.

2.3.2 Thy1.1 plasmids

Full-length Thy1.1 was cloned from pCR4-Blunt-TOPO into a pSG5 vector using standard cloning techniques. In short, the plasmids were digested with EcoRI at 37°C for 1 h and ran on a 1 % agarose gel with the addition of SYBR Safe DNA Stain for 1 h at 150 V. Bands of interest were excised from the gel and extracted with Qiagen Gel Extraction Kit, according to manufacturer's protocol. Dephosphorylation of the vector and subsequent ligation were done with Rapid DNA Dephos & Ligation Kit (Roche) in 1:3 vector: insert molar ratio. DH5α competent cells were transformed with the ligation reaction and plated on LBampicillin plates. Few colonies were picked, expanded and submitted to plasmid isolation with MidiPrep Kit (GenElute HP, Sigma). The clones were screened with Apal digestion, and the correct clone was confirmed by Sanger sequencing with T7 promoter universal primer (Macrogen).

Full-length Thy1.1 was cloned from pCR4-Blunt-TOPO into pcDNA3.1(+) with EcoRI and Apal two-step digestion, after which the above-described cloning procedure was followed. The correct clone was confirmed by Sanger sequencing with T7 promoter and BGH-R universal primers (Macrogen).

GPI anchor of Thy1 was replaced with MHC-1 transmembrane domain in the following way. Thy1.1 propeptide, which is removed when GPI is attached to Cys130 in the endoplasmic reticulum, was replaced with a part of MHC-1 molecule (Uniprot ID P01900) consisting of the connecting peptide, transmembrane domain and cytoplasmic region. pcDNA3.1(+)_Thy1.1-MHC-1 plasmid was *de novo* synthesised (Biomatik). Thy1.1-MHC-1 was cloned into a pSG5 vector using standard cloning techniques described above with EcoRI and BglII restriction enzymes in two-step digestion. The correct clone was confirmed by Sanger sequencing (University of Dundee). The amino acid sequence of the designed construct is given in **supplementary table 2**.

2.3.3 Cell culture

The B16-OVA cells with intracellular OVA were a kind gift from Ton Schumacher (The Netherlands Cancer Institute)¹¹⁴. They were cultured in IMDM medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco) and 50 µM 2-mercaptoethanol (Gibco). CHO.K1 cells (ATCC) were cultured in DMEM/F12 medium (Gibco) supplemented with 5% New Born Calf Serum (Biowest) and 1% penicillin/streptomycin (Gibco).

2.3.4 Generation of B16-OVA-Thy1.1 stable cell line

For transfection with pcDNA3.1(+)_Thy1.1, electroporation was used. In short, 5 million B16-OVA cells suspended in serum- and antibiotic-free medium were incubated with 10µg of pcDNA3.1(+)_Thy1.1 or empty vector for 10 min at room temperature. Subsequently, the cells were electroporated with a 250 V shock and a 975µF capacitor. After 5 min of resting time, 1ml of complete medium was added to the cells, and they were seeded in 60 mm Petri dishes in the final volume of 5 ml. 48h after transfection, 0.5 mg/mL of geneticin was added to the culture medium, and the cells were grown under geneticin pressure for 10-14 days. Selected cells were stained with 2µg/mL of PE anti-Thy1.1 antibody (OX7 clone, Biolegend #202524) and single-cell sorted into 96-well plates containing the selection medium with geneticin. Thy1.1 expression was regularly monitored by flow cytometry with the antibody mentioned above on FACSCanto. The clones with the highest Thy1.1 expression were expanded.

The transfection with pSG5 plasmids was done with FuGENE HD. The cells were co-transfected with 1.5 µg of pSG5 plasmid with the insert of interest and 0.5 µg of pLXSP plasmid coding for

puromycin resistance with FuGENE HD reagent (Promega) in 6:1 FuGENE: DNA ratio. Briefly, the DNA was diluted in OptiMEM medium, after which FuGENE HD was added, and the mixture was incubated for 10 min at room temperature. The transfection mixture was added dropwise to the cells at 80% confluency. 24 h after transfection, 3 µg/mL of puromycin was added to the culture medium, and the cells were grown under puromycin pressure for 10-14 days. Selected cells were stained with 2µg/mL of PE anti-Thy1.1 antibody (OX7 clone, Biolegend #202524) and single-cell sorted into 96-well plates containing the selection medium with puromycin. Thy1.1 expression was regularly monitored by flow cytometry with the antibody mentioned above on FACSCanto. Positive clones were expanded and the one showing stable Thy1.1 expression even after puromycin retrieval was selected for the *in vivo* study.

2.3.5 Thy1.1 transient transfection and cell ELISA

An amount of 24 µg of pcDNA3.1(+)-Thy1.1 plasmid was transfected into CHO.K1 cells (10 mm Petri dish, 80% confluent) using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. The following day, cells were plated into a 96-well plate (50000 cells/well). Two days after transfection, binding ELISA was performed. The cell supernatant was discarded, and either anti-Thy1.1 IgE, IgG2a or IgG1 were added in serial dilutions. The incubation was performed at room temperature for 1 h. Next, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000) or goat anti-mouse IgG Fc-HRP (Jackson Immuno Research 1:5000) in 1:1 1% BSA PBS/PBST were added for 45 min at room temperature. Immunoreactivity was visualised with TMB Stabilized Chromogen (Invitrogen). Reactions were stopped after 15 min with 0.5M H₂SO₄, and absorbances were read at 450 nm and 620 nm. All samples were tested in duplicate.

2.3.6 OT-1 activation

Fresh spleens of OT-1 mice were used for splenocyte isolation. The spleens were mashed through a 70 µm cell strainer, after which the Red Blood Cell Lysing Buffer (Hybri-Max, Sigma) was used to remove any erythrocytes. The splenocytes were plated at the density of 0.5 million cells/ml in 12-well plates (1ml/well). They were cultured in IMDM medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 50 µM 2-mercaptoethanol (Gibco)

and 2µg/mL OVA peptide (SIINFEKL). 48h later (day 2), the cells were subcultured 1:2. On day 3, the activated OT-1 cells were washed with PBS and injected intravenously via tail.

2.3.7 Mice

OT-1 mice were obtained from Rose Zamoyska (University of Edinburgh). The experiments were conducted with age-matched, 6–10 week old female mice on a C57BL/6 background purchased from Charles River. Experiments were carried out under the project license of Dr Dietmar Zaiss (PPL: PP7488818) in Ashworth Laboratories 3, Level 5, animal facility (Kings Buildings, Edinburgh). The animal experiments were approved by The University of Edinburgh.

2.3.8 *In vivo* study

50 000 B16-OVA-Thy1.1-MHC-1 cells were subcutaneously injected into the right flank. Antibody treatment consisted of either 200 µg anti-Thy1.1 IgG2a or 200 µg anti-Thy1.1 Ig1 or 10 µg anti-Thy1.1 IgE (all in house produced as described above). IgGs were administered intraperitoneally, whereas IgE was administered intravenously. The antibodies were injected on days 7, 13 and 17. Some mice received the adoptive cell transfer of 0.25 million activated OT-1 cells in PBS intravenously on day 13. The tumour size was measured regularly with a calliper. The mice were sacrificed when the tumours reached 10 mm in diameter or at the first sign of ulceration or if significant weight loss was observed (> 20% of initial weight). Tumour volume was calculated by the modified ellipsoidal formula: $V = \frac{1}{2} (\text{Length} \times \text{Width}^2)$.

2.3.9 Statistical analysis

Statistical analysis was performed in GraphPad Prism software. Survival was evaluated with the Mantel-Cox test. P-values of ≤0.05 were considered statistically significant. ns = P>0.05, * = P≤0.05

2.4 Results

2.4.1 Production, purification and quality control of anti-Thy1.1 antibodies

Anti-Thy1.1 IgG1, IgG2a and IgE antibodies were produced at our partner institution in the Netherlands (Aduro Biotech). The starting point was OX7 hybridoma (anti-Thy1.1 IgG1) which was sequenced in order to obtain heavy and light chain variable domain sequences (VH, VL). Next, we designed chimeric anti-Thy1.1 mIgE and mIgG1 heavy chains by combining the VH with the known sequences of the constant domains of murine IgE or IgG1 (CHs). Just between VH and CH domains, a unique restriction site (AfeI) was introduced, allowing us to change the isotypes by cloning. For the light chain construct, the sequence obtained by sequencing was used without any change (kappa isotype). The same light chain can be used for different heavy chain constructs (IgG1, IgG2a and IgE). The DNA sequences encoding those constructs were *de novo* synthesised in pcDNA3.1(+) expression vector surrounded by HindIII and EcoRI unique restriction sites. The missing antibody isotype construct (IgG2a) was made by cloning the IgG2a constant heavy domain from an available plasmid at Aduro, as represented in **figure 2.1**. Similarly, the IgG2a heavy chain with LALA-PG mutations was cloned from an available anti-TNFR antibody following the same approach.

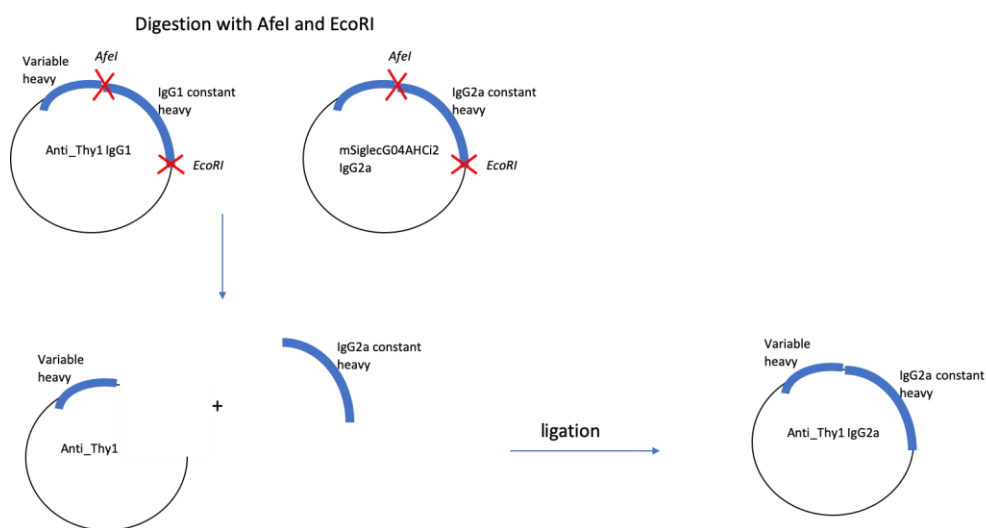


Figure 2.1. Schematic representation of the cloning strategy used for isotype switching. Our antibody construct design introduced AfeI and EcoRI unique restriction sites surrounding the CH domains. This allowed us to switch isotypes by simple cloning. This approach was used to obtain the anti-Thy1.1 IgG2 construct by cloning the IgG2 CH from an available plasmid indicated on the scheme into the plasmid coding for the anti-Thy1.1 VH domain.

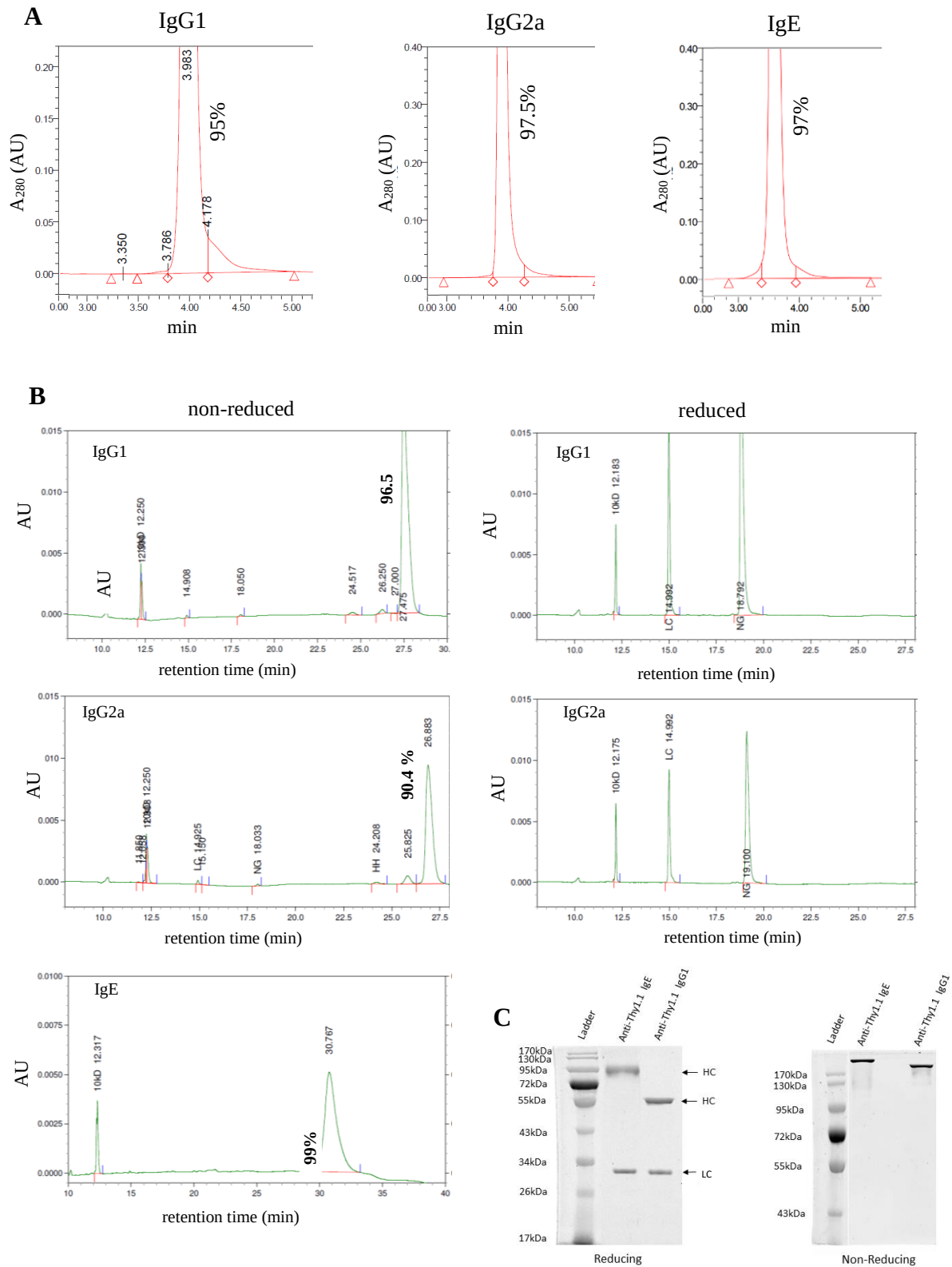


Figure 2.2. Quality control of anti-Thy1.1 antibodies. (A) Monomericity was evaluated with UPLC-SEC, monomer percentage is shown; (B) CE-SDS under non-reducing conditions and purity percentage (left) and under reducing conditions (right); (C) SDS-PAGE was used for IgE evaluation as a complementary method, since CE-SDS was not optimised for IgE.

The antibodies were produced on a large scale and purified by either protein A affinity chromatography (IgG1 and IgG2a antibodies) or thiophilic chromatography (IgE). The quality of produced antibodies was studied by different methods. Size-exclusion ultra-performance liquid chromatography (SE-UPLC) was used to test monomericity, which was >95% for all three antibodies (**Fig. 2.2 A**). Next, the purity was tested by capillary electrophoresis sodium dodecyl sulphate (CE-SDS). Since CE-SDS was not optimised for IgE, we also included SDS-PAGE to confirm the correct molecular weights and purity of IgE. The analysis under non-reducing conditions confirmed the expected molecular weights and indicated that a high purity (>90%) was reached in all samples (**Fig. 2.2 B (left) and C**). Furthermore, only HC and LC were observed under reducing conditions, confirming the correct sample composition (**Fig. 2.2 B (right) and C**). Taken together, the produced antibodies complied with high-quality standards regarding monomericity and purity. In addition, we confirmed that the antigen binding was preserved in binding ELISA with Thy1.1 expressing CHO cells (**Fig. 2.3**). No difference in binding was observed between different isotypes.

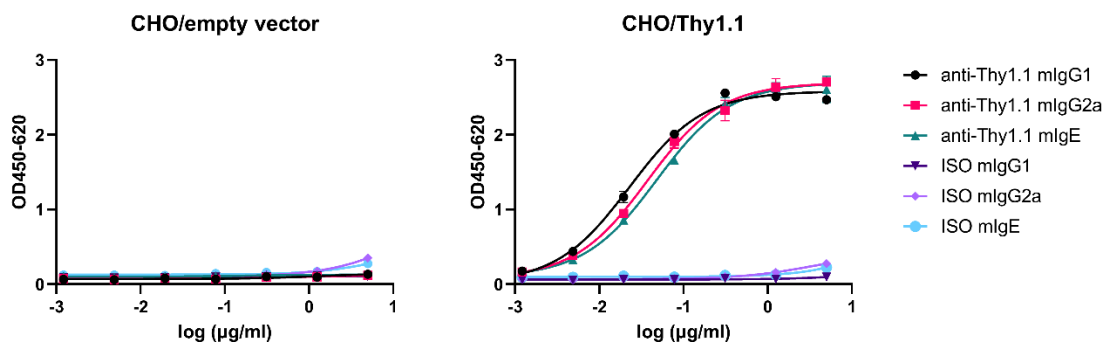


Figure 2.3. Cell binding ELISA of anti-Thy1.1 antibodies. The binding of anti-Thy1.1 IgG1, IgG2a and IgE was tested on CHO cells transiently transfected with an empty vector (left) or Thy1.1 (right). Isotype controls were used for each antibody isotype. Mean + SD of duplicates are shown.

2.4.2 Achieving stable Thy1.1 expression on B16-OVA cells

The next step of this study was to establish a B16-OVA tumour cell line stably expressing Thy1.1. To this end, I transfected the B16-OVA cells with pcDNA3.1(+)-Thy1.1 vector and grew them on a geneticin selection medium for ten days. Next, the single-cell sorting was performed, and the clones were expanded and screened for Thy1.1 expression by FACS. However, no good clones were observed, and the cells were losing the Thy1.1 expression over time (**Fig. 2.4**).

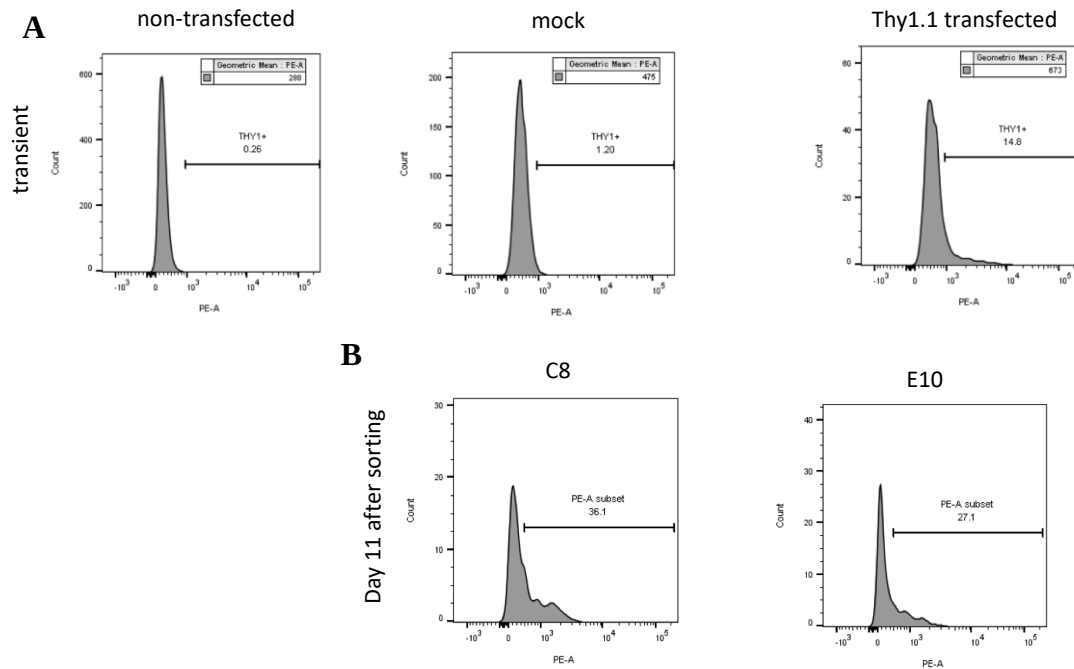


Figure 2.4. FACS analysis of Thy1.1 expression on B16-OVA cells after transfection with pcDNA3.1(+)-Thy1.1. B16-OVA cells were transfected with pcDNA3.1(+)-Thy1.1, selection agent (geneticin) was added 48h after transfection and single-cell sorting was performed after 10 days of growing the cells in the selection medium. Thy1.1 expression was regularly tested by FACS. (A) Transient expression 24h after transfection. (B) The expression on two selected clones 11 days after single-cell sorting.

We thought that the problem could be explained by the methylation of the CMV promoter present in our vector, leading to its silencing. Therefore, I decided to use the pSG5 vector with SV40 promoter instead. The Thy1.1 sequence was cloned into the pSG5 vector and co-transfected with pLXSP plasmid coding for puromycin resistance into B16-OVA cells. However, this attempt failed as well, and we observed a similar loss of Thy1.1 expression over time (**Fig. 2.5**).

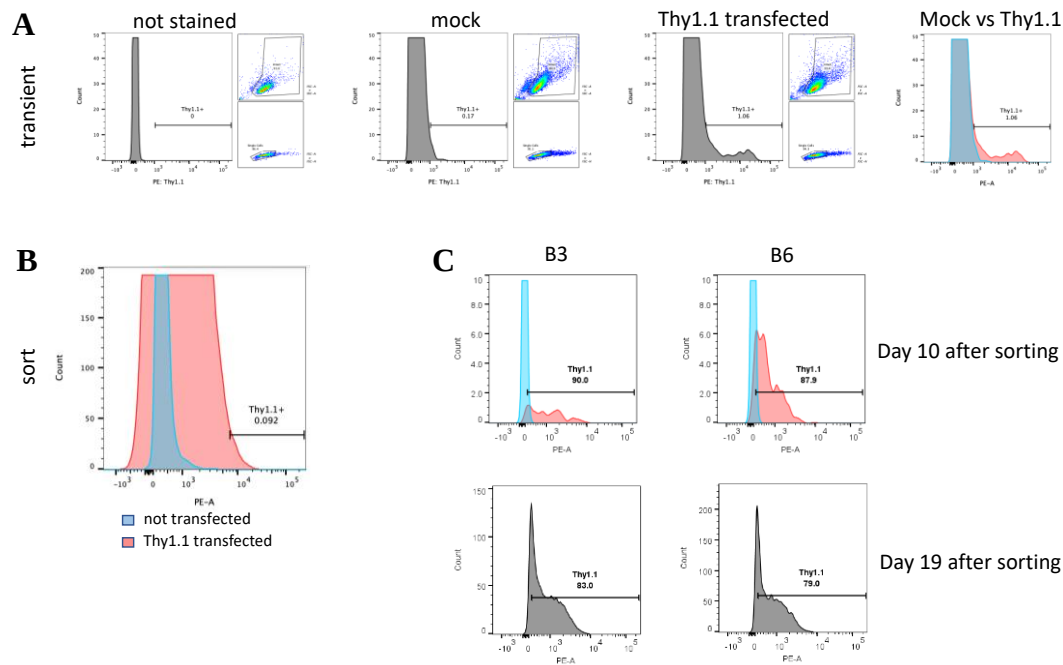


Figure 2.5. FACS analysis of Thy1.1 expression on B16-OVA cells after transfection with pSG5-Thy1.1. B16-OVA cells were co-transfected with pSG5-Thy1.1 and pLXSP, selection agent (puromycin) was added 24h after transfection and single-cell sorting was performed after at least 10 days of growing the cells in the selection medium. Thy1.1 expression was regularly tested by FACS. (A) Transient expression 24h after transfection. (B) Expression at single-cell sorting. (C) Expression on two selected clones 10 and 19 days after single-cell sorting.

Having tried to express Thy1.1 under two different promoters without success, we suspected the problem might lie in Thy1.1 itself. Thy1.1 is a glycosylphosphatidylinositol (GPI) anchored cell surface protein, and it is, therefore, susceptible to the cleavage of GPI anchor by phospholipase C¹¹⁵ (**Fig. 2.6 A**). This may cause the loss of expression in B16-OVA cells. To overcome this, we replaced the GPI anchor of Thy1.1 with a murine MHC-1 transmembrane domain (**Fig. 2.6 B**). MHC-1 was chosen as both Thy1.1 and MHC-1 belong to the immunoglobulin superfamily. Finally, B16-OVA cells co-transfected with pSG5_Thy1.1-MHC-1 and pLXSP showed stable Thy1.1 expression under puromycin selection, and I was able to expand single-cell clones. Since this cell line was intended to be used *in vivo* where no puromycin pressure would be present, I also tested the stability of Thy1.1 expression under puromycin-free conditions *in vitro* for almost three weeks. B16-OVA-Thy1.1 clone showed stable Thy1.1 expression even after puromycin removal, confirming that the clone was very stable (**Fig. 2.6 C-E**). Of note, the expression was studied using the same anti-Thy1.1 antibody

clone (OX7) that we used for the *in vivo* studies afterwards. Thus, the replacement of the Thy1.1 transmembrane domain did not affect the binding of the anti-Thy1.1 antibody.

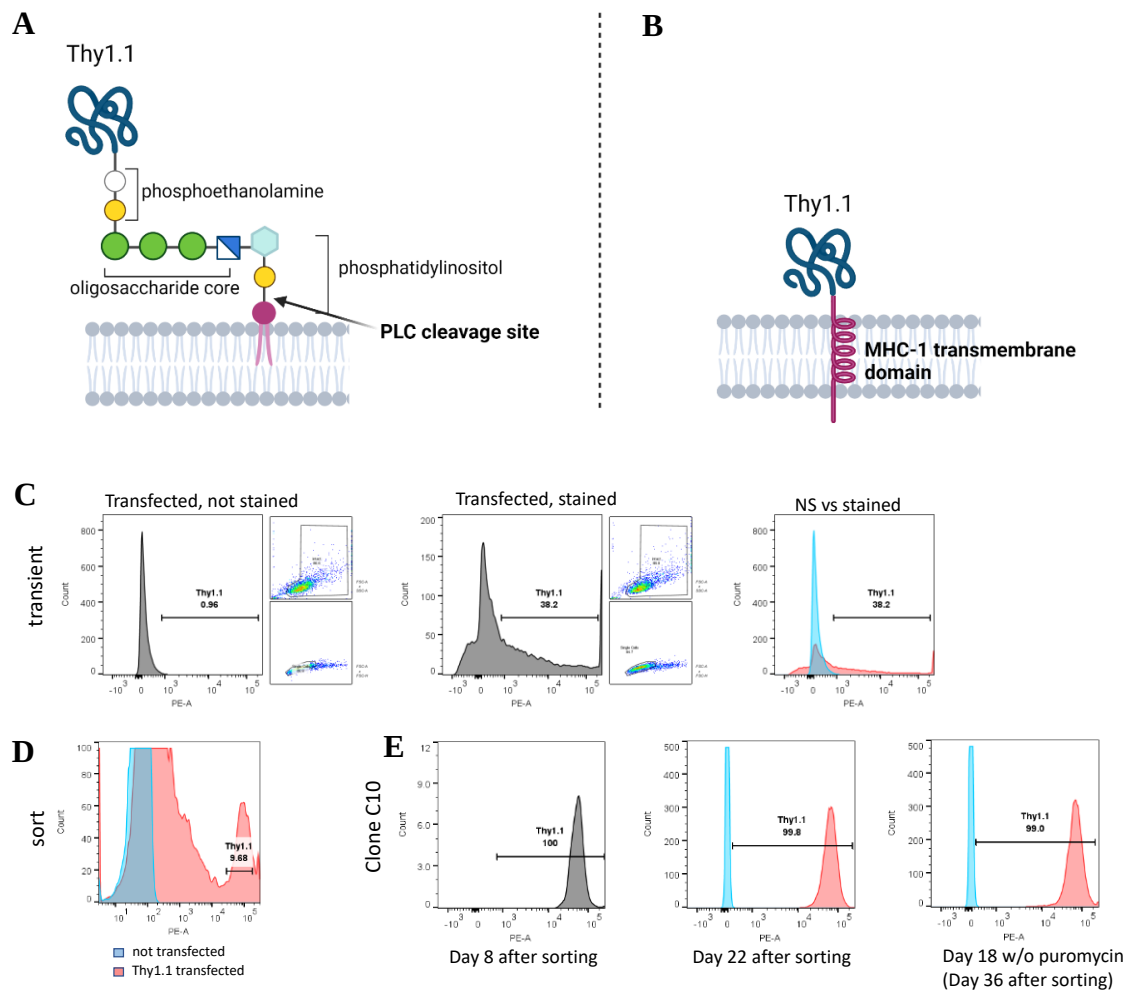


Figure 2.6. Successful expression of Thy1.1-MHC-1 on B16-OVA cell. B16-OVA cells were co-transfected with pSG5-Thy1.1-MHC-1 and pLXSP, selection agent (puromycin) was added 24h after transfection and single-cell sorting was performed after at least 10 days of growing the cells in the selection medium. Thy1.1 expression was regularly tested by FACS. Schematic representation of (A) Thy1.1 with its GPI anchor and (B) the designed construct in which the GPI anchor has been replaced with MHC-1 transmembrane domain. (C-E) FACS analysis of Thy1.1 expression on B16-OVA cells after transfection with pSG5-Thy1.1_MHC-1. (C) Transient expression 24h after transfection. (D) Expression at single-cell sorting. (E) Expression on the selected clone on the indicated days.

2.4.3 Anti-Thy1.1 IgG2a shows superior tumour control to its IgG1 and IgE homologues *in vivo*

Once the Thy1.1 target cell line and Thy1.1-targeting antibodies were ready, we tested the ability of different antibody isotypes to control the tumour growth in a syngeneic mouse

model. To this end, C57BL/6 mice were subcutaneously injected with B16-OVA-Thy1.1 cells and treated with either anti-Thy1.1 IgG2a, IgG1 or IgE. We chose to use a different dose and route of administration for IgE compared to standardly used 200µg i.p. for IgG antibodies. IgE requires lower doses due to its high affinity for FcεRI. Thus, we decided to inject 10µg IgE i.v. as this approach is commonly used to achieve systemic sensitisation of mice in allergy studies¹¹⁶.

In addition, the antibodies were also tested in combination with the adoptive cell transfer of activated OT-1 cells. B16-OVA tumours are characterised by an immune-suppressive tumour microenvironment dominated by T_{regs}. It has been shown that depletion of intratumoral T_{regs} offers tumour protection when combined with the GVAX vaccine due to enhanced activation of CD8⁺ T cells^{117,118}. Thus, OT-1 efficacy in this setup can be inversely correlated with T_{reg} function. With the B16-OVA cell line that we used, OT-1 monotherapy is usually ineffective when given after day seven post tumour implantation (unpublished observation from our group). Thus, we injected the OT-1 cells at a later stage of tumour development when they can no longer control the tumour growth due to an established immune-suppressive tumour microenvironment. This allowed us to test whether our antibodies attenuate this immune-suppressive TME and rescue OT-1 efficacy. The experiment scheme is represented in **figure 2.7**.

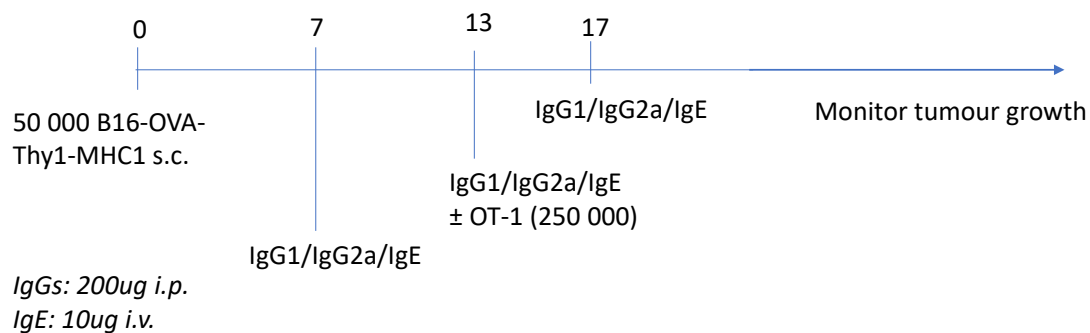


Figure 2.7. Experimental scheme of the antibody isotype comparison in the B16-OVA-Thy1.1 model.

Our results show that OT-1 treated mice had similar outcomes to those that did not receive OT-1 adoptive cell transfer (**Fig. 2.8 A**). Therefore, IgG2a, IgG1, and IgE treatments could not rescue OT-1 activity in this setup. Regarding the efficacy of different isotypes, IgG2a showed superior tumour control to IgG1 and IgE (**Fig. 2.8 A-B**). Whereas all IgG1- (10/10) and IgE- (12/12) treated animals reached the human endpoint by day 49, 50% (6/12) of IgG2a-treated

mice were still alive at day 60, with very small tumours or no tumours at all. Median survival was 24 days for IgG1 and 26 days for IgE, compared to 48 days for IgG2a (**Fig. 2.8 C**).

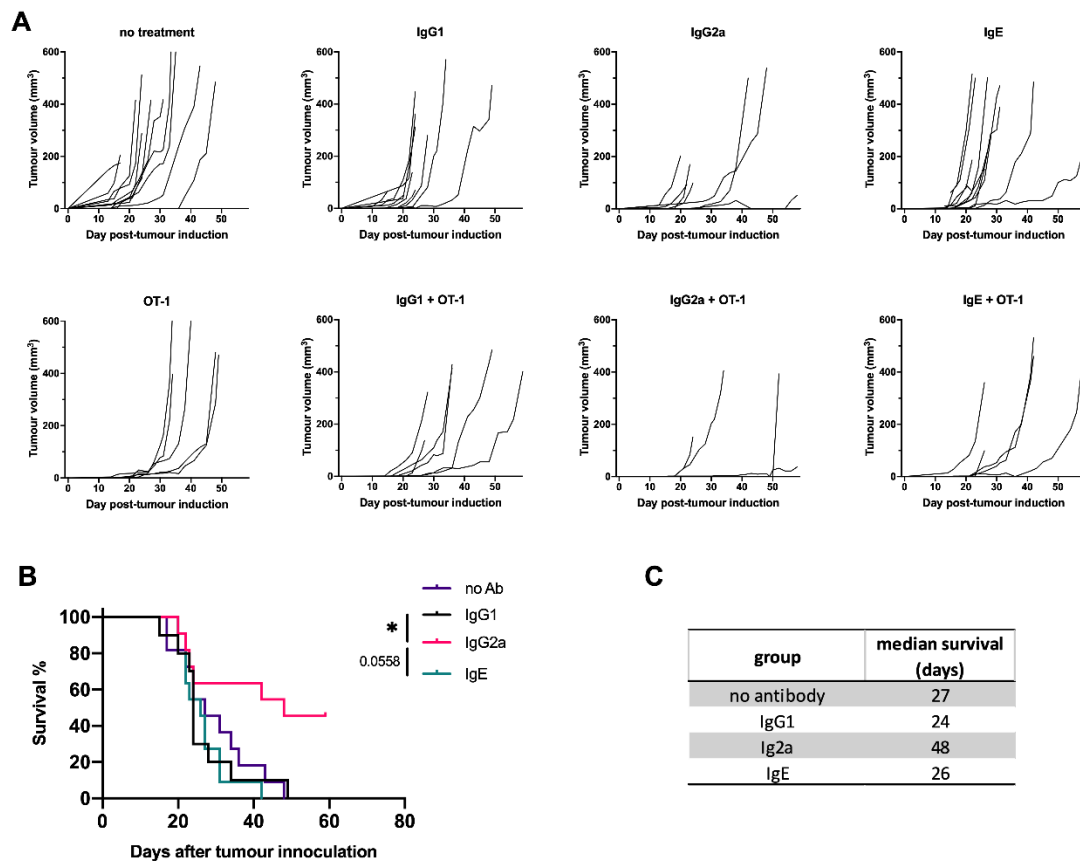


Figure 2.8. Superior tumour growth control of anti-Thy1.1 IgG2a *in vivo*. C57BL/6 mice were subcutaneously injected with 50 000 B16-OVA-Thy1.1 cells in the flank and were treated with anti-Thy1.1 IgG1, IgG2a or IgE antibodies. Some mice received the combination treatment consisting of anti-Thy1.1 antibodies and adoptive cell transfer of activated OT-1 cells. (A) Tumour growth curves. (B) Survival analysis. (C) Median survival in days. Statistical significance was calculated with the Mantel-Cox test. * = $P \leq 0.05$

A (top), B and C: $n=10-12$, combined data of two independent experiments. A (bottom): $n=5-6$, the experiment was done once.

2.4.4 Superior anti-tumoral effects of anti-Thy1.1 IgG2a are Fc-mediated

Next, we wanted to confirm that the tumour control we achieved with the IgG2a isotype was due to Fc-mediated effector function. To show that, we introduced LALA-PG mutations in the constant domain of the IgG2a heavy chain. LALA-PG mutations have been shown to significantly reduce the binding of both human and murine IgG antibodies to Fcγ receptors¹¹⁹. In the case of mIgG2a, the binding to FcγRI, II and IV is completely destroyed, while the binding

to FcγRIII is reduced more than 50-fold. In addition, LALA-PG mutants show decreased C1q binding and C3 fixation in murine serum.

When we compared the anti-Thy1.1 IgG2a and IgG2a-LALA-PG *in vivo* following the same approach as shown in **figure 2.7**, we observed a complete loss of efficacy with the Fc-silenced antibody (**Fig. 2.9**). Whereas IgG2a survival rate was around 50%, as observed in our previous experiment, all the mice treated with IgG2a-LALA-PG reached the endpoint by day 39. This result clearly shows that the observed anti-tumour effect of the anti-Thy1.1 IgG2a antibody was Fc-mediated.

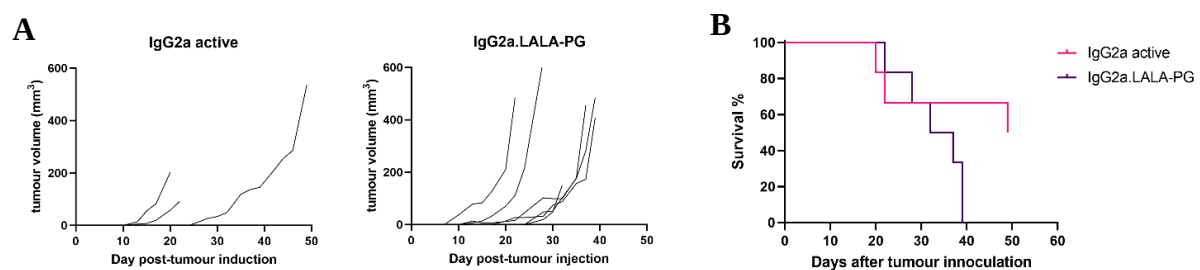


Figure 2.9. *In vivo* tumour control is lost when IgG2a Fc tail is silenced. C57BL/6 mice were subcutaneously injected with 50 000 B16-OVA-Thy1.1 cells in the flank and were treated with anti-Thy1.1 IgG2a (active) or anti-Thy1.1 IgG2a-LALA-PG (Fc silent) antibody. (A) Tumour growth curves. (B) Survival analysis. n=5-6, the experiment was done once.

This experiment has been done by a fellow PhD student Aina Segués while I was away finishing a different project at the partner institution in Spain.

2.5 Discussion

IgE isotype has shown superior anti-tumour effects to commonly used IgG in multiple preclinical studies. However, those studies have not addressed the potential outcome of IgE-mediated activation of MCs and basophils on tumour development. Since IgE can induce extremely potent immune reactions through these cell types, diverting them against tumour cells could have therapeutic benefits. Mice represent a good model for addressing this question, as their FcεRI expression is limited to MCs and basophils¹³.

In this chapter, I directly compared the therapeutic potential of murine IgG2a, IgG1 and IgE of the same specificity targeting a surface tumour antigen (Thy1.1). Wild type mice bearing syngeneic B16-OVA-Thy1.1 tumours were used for this purpose. In addition, we combined the antibody treatment with OT-1 adoptive cell transfer, which, as monotherapy, is usually not effective in rejecting the B16-OVA tumours due to immune-suppressive TME¹¹⁴. Thus, by studying the potential synergic effect, we were able to evaluate if different antibody isotypes revert that immune suppression and induce favourable changes in the TME. We were particularly interested if IgE could mediate such an effect by inducing the T_{reg} suppression via histamine released from degranulating MCs.

My results show that IgE treatment did not have any effect on the tumour growth, as the growth curves and survival function were not significantly different compared to not treated mice. A similar approach has been recently used by a group at Massachusetts Institute of Technology (MIT) that showed that IgE targeting a surface tumour antigen could not successfully control the tumour growth in B16-OVA and MC-38 models in wild type mice¹²⁰. The lack of effect that we observed may be explained by the poor presence of IgE effector populations in B16-OVA tumours. We performed a histological analysis of B16-OVA tumours (presented in chapter 3, figure 3.4), which revealed that MCs (and possibly basophils) were primarily present on tumour edges. Very few actually infiltrated deeper into the tumour. Thus, targeting a surface tumour antigen with an IgE antibody may not be optimal for MC/basophil activation due to their particular localisation within the B16-OVA tumours. In the following chapters, we address this question by targeting secreted and intracellular tumour antigens. By being soluble, they have better chances of reaching MCs at the tumour edges, and they more closely resemble allergens as typical IgE targets. Of note, although the

histological analysis was not done for the B16-OVA-Thy1.1 clone, we do not think that expressing Thy1.1 would have caused any significant difference regarding MC/basophil presence.

IgG2a is well-known as the most active IgG subclass in mice due to its high A/I ratio. Nevertheless, direct comparisons of different antibody isotypes of the same specificity in cancer settings are scarce. For example, Dahan et al. showed that the anti-PD-L1 IgG2a antibody is superior to IgG1 in MC38 and B16-OVA tumour models¹²¹. However, PD-L1 expression is not restricted to tumour cells, making it challenging to extrapolate their result to exclusively tumour antigen-targeting mAbs. Here, by using Thy1.1 as a model antigen exclusively present on tumour cells, we confirmed that only the IgG2a isotype significantly improves the mice survival in the B16-OVA model. Thus, we provide *in vivo* evidence that tumour antigen targeting IgG2a is superior to its IgG1 homolog in controlling tumour growth. In humans, the equivalent of mIgG2a as the most active IgG subclass would be hIgG1³. Unfortunately, the superiority of IgG2a over IgE in wild type mice cannot be translated to human settings due to substantially different FcεR cellular expression profiles in these two species.

Furthermore, we showed that neither of the tested antibody isotypes was able to rescue the efficacy of OT-1 treatment, indicating that the immune-suppressive tumour microenvironment has not been significantly altered. The potential to change the balance in the TME away from the immune-suppressive state was expected for the IgE isotype. However, as already discussed, IgE failed to induce any effect on tumour growth, potentially due to the poor presence of MCs in the tumours. Therefore, another approach would be needed to address this question. For example, MCs preloaded with IgE could be injected intratumorally to increase their numbers and presence inside the tumour. Alternatively, the IgE treatment could be combined with an intratumoral injection of an MC chemoattractant SCF. Nevertheless, as already discussed, since allergens are typically soluble, we believe that switching to a soluble tumour antigen would be a more physiological way of IgE-mediated MC activation. That approach is presented in the following chapters of this thesis.

Finally, our study offers an additional technical contribution in terms of overcoming difficulties with Thy1.1 expression. The loss of Thy1.1 expression over time in B16-OVA cells

has been reported before, even in retrovirally transduced cells¹²². This strongly supports our hypothesis that Thy1.1 expression is lost due to GPI cleavage and not promoter-related issues. We offer the solution to this problem by replacing that GPI anchor with the MHC-1 transmembrane domain. Since Thy1.1 is not usually expressed on mouse cells (except for a few particular mouse strains), it can be an excellent marker for detecting transfectants. This is of particular interest if the protein of interest is expressed intracellularly. In that case, using Thy1.1 as a surrogate marker on the cell surface would allow easy expression evaluation and selection of positive clones. Importantly, Thy1.1 is not immunogenic when injected into recipient mice.

Limitations

There are a few limitations of the work presented in this chapter that I would like to discuss. Firstly, although using the Thy1.1 as a model antigen comes with important advantages, such as its exclusive expression on tumour cells and non-interference with tumour signalling pathways, this approach also has a possible disadvantage. Because Thy1.1 is not essential for the tumour cells in any way, it is expected that its expression is downregulated *in vivo*. Indeed, when we analysed the tumour material at the end of the *in vivo* experiment, only about 15% of tumour cells still expressed Thy1.1 (data not shown). Although the loss of tumour antigen expression is not uncommon with antibody treatments, it is an important aspect to consider.

Secondly, although we suspect that the lack of IgE effect is due to poor contact between MCs, which are mainly localised at tumour periphery, and tumour cells, we have not confirmed the presence of administered IgE antibody in the tumour. Thus, it would be important the study the IgE biodistribution. This could be done by *in vivo* tracking of fluorescently labelled antibody using the IVIS system¹²³. Then, the IgE dose could be optimised until optimal accumulation of IgE at tumour site is achieved.

Furthermore, we cannot completely rule out the possibility that IgE did activate the MCs, but that their activation did not affect the tumour development. Evaluating local MC activation at the tumour site is technically challenging, as discussed in chapter 3 (limitations). However, since targeting a secreted tumour antigen with IgE in the same B16-OVA model had a certain biological effect (section 3.2.3), we are more confident that optimal MC activation simply did not occur with IgE against a surface target antigen.

Finally, it is important to note that our approach to overcoming Thy1.1 expression difficulties was only tested in B16-OVA cells. Thus, its validation in another cell line is still needed.

Future outlooks

We have shown that IgG2a offers superior tumour control to IgG1 and that an active Fc tail was essential for such an effect. However, it would be important to detect the immune cell population through which IgG2a mediates its anti-tumour effect. Since many IgG2a-treated mice did not develop any visible tumours, studying the tumour material would not be possible. However, depleting potential IgG effector cell populations would allow us to confirm which cell immune cells are mainly responsible for IgG2a-mediated tumour control. For this purpose, anti-NK1.1 antibody and CSF-1R inhibitors or clodronate liposomes can be used to deplete NK cells and macrophages, respectively. This experiment would be a final important confirmation of our findings. In addition, immunophenotyping of the TME and tumour-draining lymph nodes by FACS could be carried out to detect any changes in different immune cell populations induced by the treatment.

IgE did not show any effect on tumour development in this setup, and we propose that a surface tumour antigen is not the best choice to achieve IgE-mediated MC and basophil activation. Thus, in the following two chapters, we focus on soluble tumour antigens, either secreted or intracellular, in search of an optimal way to induce the wanted anti-tumour response.

Conclusions

In conclusion, in this chapter, I show that IgG2a targeting a surface tumour antigen exhibits superior tumour control to its IgG1 and IgE homologs in wild type mice. The effect was Fc-mediated as it was completely lost with the IgG2a featuring Fc silencing LALA-PG mutations. Next, neither IgG2 nor IgG1 nor IgE could rescue the OT-1 efficacy in the B16-OVA model, reflecting their inability to revert the immune-suppressive tumour microenvironment. Furthermore, IgE targeting a surface tumour antigen did not induce any effect on the tumour growth, suggesting that this is not an optimal approach to activate MCs and basophils at the tumour site. Finally, I describe a successful strategy to overcome difficulties with Thy1.1 expression B16-OVA cells by replacing its GPI anchor with the MHC-1 transmembrane

domain. This approach can have a broad application in detecting transfectants, where Thy1.1 could be used as an expression marker.

Supplementary Table 1. Amino acid sequences of anti-Thy1.1 antibodies. Signal peptides are highlighted in grey, and LALA-PG mutations in cyan.

Anti-Thy1.1 IgG2a HC
MAVLGLLFCLVTFPSCVLSEIQLQQSGPELMKPGASVKISCKASGYSTSYMDWVKQSHGKNLEWIGYIDPFN GDSYNQKFKDKATLTVDKSSSTAYMHLSSLTSEDSAVYYCARGIYYGYGGYFDYWGGQTTLTVSSAKTTAPSVY PLAPVCGDGTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVA HPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMSLSPIVTCVVVDVSEDDPDVQISWV NNVEVHTAQTQTHREDYNSTLRVVSALPIQHQQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLP PPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLSDSGSYFMYSKLRVEKKNWVERNSYS CSVVHEGLHNHHTTKSFSRTPGK
Anti-Thy1.1 IgG2a LALA-PG HC
MAVLGLLFCLVTFPSCVLSEIQLQQSGPELMKPGASVKISCKASGYSTSYMDWVKQSHGKNLEWIGYIDPFN GDSYNQKFKDKATLTVDKSSSTAYMHLSSLTSEDSAVYYCARGIYYGYGGYFDYWGGQTTLTVSSAKTTAPSVY PLAPVCGDGTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVA HPASSTKVDKKIEPRGPTIKPCPPCKCPAPNAAGGPSVFIFPPKIKDVLMSLSPIVTCVVVDVSEDDPDVQISWV NNVEVHTAQTQTHREDYNSTLRVVSALPIQHQQDWMSGKEFKCKVNNKDLGAPIERTISKPKGSVRAPQVYVLP PPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLSDSGSYFMYSKLRVEKKNWVERNSYS CSVVHEGLHNHHTTKSFSRTPGK
Anti-Thy1.1 IgG1 HC
MAVLGLLFCLVTFPSCVLSEIQLQQSGPELMKPGASVKISCKASGYSTSYMDWVKQSHGKNLEWIGYIDPFN GDSYNQKFKDKATLTVDKSSSTAYMHLSSLTSEDSAVYYCARGIYYGYGGYFDYWGGQTTLTVSSAKTTAPSVY PLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSSETVTN VAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLITLTPKVTCVVVDISKDDPEVQFSWFVDDVE VHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAFAPIEKTISKTKGRPKAPQVYTIPPPKEQ MAKDKVSLTCMITDFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNQVQSNWEAGNTFTCSVL HEGLHNHHTKSLSHSPGK
Anti-Thy1.1 IgE HC
MAVLGLLFCLVTFPSCVLSEIQLQQSGPELMKPGASVKISCKASGYSTSYMDWVKQSHGKNLEWIGYIDPFN GDSYNQKFKDKATLTVDKSSSTAYMHLSSLTSEDSAVYYCARGIYYGYGGYFDYWGGQTTLTVSSASIRNPQLY PLKPCGTASMTLGCLVKDYFPNPVTVTWYSDSLNMSTVNFALGSELKVTTSQVTSWGKSAKNFTCHVTHPP SFNESRTILVRPNITEPTLELLHSSCDPNAFHSTIQLYCFIYGHILNDVSVSWLMDREITDTLAQTVLIKEEGKLA STCSKLNITEQQWMSESTFTCKVTSQGVLDYLAHTRCPDHEPRGVITYLIPPSPLDLYQNGAPKLTCLVVDLESEK NVNVTWNQEKTSVSASQWYTKHHNNATTSITSILPVVAKDWIEGYGYQCIVDHPDFPKPIVRSITKTPGQRSA PEVYVFPPPEEESDKRTLTLCLIQNFPEDISVQWLGDGKLISNSQHSSTTTLKSNQNGFFIFSRLEVAKTLWT QRKQFTCQVIHEALQKPRKLEKTISTSLGNTSLRPS
Anti-Thy1.1 LC
MSVLTVLALLLWLTGARCDIVLTQSPASLAVSLGQRATISCRASDSVDSFGNSFMHWFQQKPGQPPKLLIYR ASTPESGIPARFSGSGSRDFTLTISPVEADDVATYYCQSIQEDPFTFGGGTKLEIKRADAAPTYSIFPPSSEQLTSG GASVVCFLNNFYPKDINVWKIDGSRQNGVLNSWTDQDSKDSTYSMSSTLTLTDEYERHNSYTCEATHKST SPIVKSFNRENC

Supplementary Table 2. Amino acid sequence of designed Thy1.1-MHC-1 construct

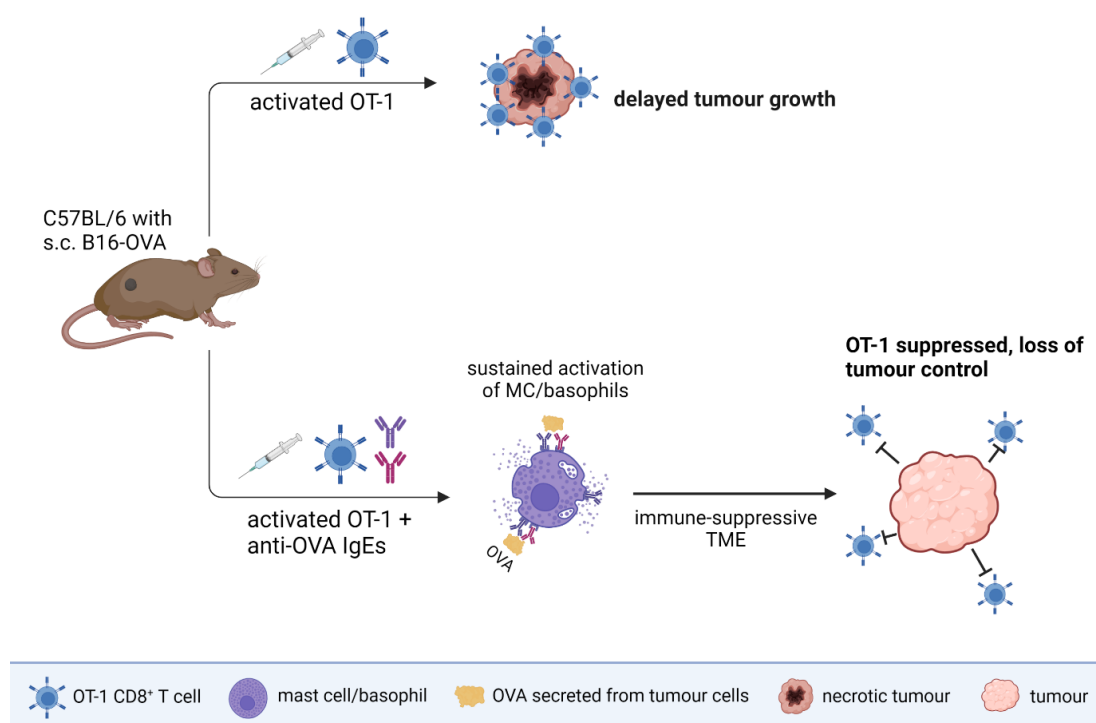
Thy1.1-MHC-1
MNPVISITLLSVLQMSRGQRVISLTACLVNQNLRLDCRHENNTNLPIQHEFSLTREKKKHVLSGTLGVPEHTYRS RVNLFSDRFIKVLTLANFTTKDEGDYMCRLVSGQNPTSSNKTINVIRDKLVKCGKEEPSSTKTNTVIIAVPVVLG AVVILGAVMAFVMMRRRNTGGKGGDYALAPGSQSSDMSLPDCKV

Legend: Signal peptide – Thy1.1 without its propeptide – connecting peptide – transmembrane domain of MHC-1 – cytoplasmic domain of MHC-1

3 Chapter 3: Targeting a secreted tumour antigen with IgE treatment

3.1 Abstract

To more closely resemble the nature of allergic response where allergens are mostly soluble proteins, in this chapter, we have chosen to target a secreted tumour antigen (OVA) with anti-OVA IgE treatment in the B16-OVA model. Our *in vitro* data indicated that the combination of two different anti-OVA IgE antibodies was needed to induce MC degranulation with B16-OVA cell supernatant. Thus, I used the same combination of IgE antibodies in the mouse model. My results show that IgE treatment alone did not affect tumour growth. However, it abolished the protective effect of OT-1 adoptive cell transfer. Thus, we suggest that the sustained presence of a secreted tumour antigen led to chronic IgE-mediated activation of effector cells and unfavourable changes in the TME. The graphical abstract of the work described in this chapter and the proposed hypothesis are given below.



C57BL/6 mice were subcutaneously injected with B16-OVA cells that secrete OVA. The mice that were treated with OT-1 adoptive cell transfer had significant delay in tumour growth and better survival, whereas the addition of IgE treatment completely abolished the protective effect of OT-1. We believe this is due to sustained activation of IgE effector cells and creation of immune-suppressive TME.

3.2 Introduction

The presence of allergies and high IgE serum levels have been correlated with overall lower cancer incidence and better prognosis^{30,31}. In addition, it was reported that intratumoral MC degranulation could eradicate tumours⁹⁴. Therefore, IgE-induced MC degranulation looks like a promising approach in cancer treatment. However, it has also been suggested that chronic inflammation at the primary allergy site has pro-tumorigenic effects³¹, many of which can be MC-mediated. For example, MCs release multiple mediators involved in tissue remodelling, immunosuppression and angiogenesis⁸⁰. A similar dual role has also been described for basophils⁹⁸. Thus, MCs and basophil activation in tumour settings can be a double-edged sword, and we are still searching for an optimal way to promote MC and basophil cytotoxicity without inducing their cancer-promoting effects. We hypothesise that antigen localisation and type of exposure may result in different outcomes of IgE-mediated MC and basophil activation, ultimately leading to either beneficial or detrimental effects in cancer therapy.

In the previous chapter, I showed that targeting a surface tumour antigen with IgE antibodies in wild type mice induced neither detrimental nor beneficial effects on tumour growth. These results strongly suggest that IgE effector cells (MCs and basophils) were probably not in close proximity to tumour cells expressing the antigen; thus, IgE possibly failed to activate them and mediate any effect. Therefore, in this chapter, I decided to target an antigen secreted from the tumour cells and, in such a way, improve the chances of MCs and basophils encountering it. Secreted tumour antigens such as prostate specific antigen (PSA) and carcinoembryonic antigen (CEA) are widely used as tumour markers and could represent a good physiological target in humans¹²⁴. Since most allergens are soluble proteins, a secreted protein would also more closely mimic the pathophysiology of the allergic response. In addition, in order to evaluate the potential effect of MC activation on TME and immunosuppression, IgE treatment was combined with adoptive CD8⁺ T cell transfer. To this end, I used a well-known subcutaneous B16-OVA model and activated OT-1 T cell treatment.

First, the anti-OVA IgE antibody was produced, and its antigen-binding properties were validated. Next, we show that one anti-OVA IgE antibody alone is insufficient to achieve MC degranulation against OVA secreted from B16-OVA cells. Thus, I combined two different anti-OVA IgE antibodies in the *in vivo* study to induce effector cell degranulation. In this setup, IgE

treatment completely abolished the protective effect of OT-1 ACT. These results suggest that targeting a secreted antigen may have led to a sustained IgE-mediated MC and basophil activation, resembling the chronic inflammation conditions present at primary allergic sites. The changes in the TME may explain the loss of efficacy of CD8⁺ T cells in clearing the tumour, but further studies are needed to confirm this hypothesis.

3.3 Materials and methods

3.3.1 Cell culture

B16-OVA cells with full-length secreted OVA were kindly gifted by Pedro Berraondo (University of Navarra, CIMA). B16-OVA cells with truncated intracellular OVA were kindly gifted by Ton Schumacher (The Netherlands Cancer Institute)¹¹⁴. All B16 lines were cultured in IMDM medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 50 μ M 2-mercaptoethanol (Gibco). For the B16-OVA cell line that secretes OVA, 400 μ g/ml geneticin was used as a selection medium.

3.3.2 Production of anti-OVA IgE antibody (clone 2C6)

2C6 hybridoma cells were kindly provided by Prof. Lester Kobzik (Harvard)¹²⁵. Anti-OVA IgE antibody was obtained from 2C6 hybridoma as described before¹¹³. Briefly, the hybridoma cells were grown in serum-free medium for seven days, after which the supernatant was collected and the antibody purified with thiophilic interaction chromatography and preparative SEC.

3.3.3 Biotinylation of anti-OVA IgE antibody

Biotinylation was performed with EZ-Link NHS LC-Biotin (Thermo Fischer Scientific #21336) per the manufacturer's protocol by adding 20-fold molar excess of the reagent to the antibody. The reaction was incubated for 60 min at room temperature. The unbound biotin was removed by washing the reaction solution three times with PBS in the Zeba Spin desalting column. Biotinylation was quantified with Pierce Biotin Quantitation Kit (Thermo Fischer Scientific #28005) according to the manufacturer's protocol. The assay is based on the fact that biotin has a higher affinity than HABA for avidin, so it displaces HABA from its complex with avidin, leading to an absorbance decrease. It allows the estimation of the mole-to-mole ratio of biotin to protein. Biotinylated HRP is used as a positive control.

3.3.4 Cell lysates

The cell plates were washed twice with cold PBS. 1 mL of cell dissociation buffer was added per 10 cm plate, and the plates were incubated at 37°C for 10 min. Cells were harvested in a

15 mL tube and centrifuged at 1500 rpm for 3 min at 4°C. The cells were then washed twice with cold PBS, after which the lysis was performed on ice for 30 min with RIPA lysis buffer containing protease inhibitor cocktail (Roche #4693159001). 1 mL buffer was used per 10 million cells. The lysed cells were centrifuged for 20min at 12000 rpm at 4°C. Supernatants were collected and frozen at -80°C. The protein content was quantified in the BCA assay as per the manufacturer's protocol (Pierce BCA™ Protein Assay Kit, Thermo Scientific #23225). The protein concentration was similar in each cell lysate (~1mg/mL, data not shown).

3.3.5 DotBlot

Spots where the samples will be applied were marked with a pencil on an Immobilon-P membrane. Next, the membrane was laid on methanol for 15 seconds, washed in ultrapure water for 2 min and equilibrated in TBST buffer for 5 min. The stack was assembled as follows: paper towels, dry filter paper, filter paper prewet in TBST and the prepared membrane. 2 µL of indicated samples were spotted onto the membrane within the pre-marked circle area. Once the samples were absorbed, the membrane was placed on a new dry filter paper and left for an hour at room temperature to dry. The membrane was blocked in 5% milk for one h and incubated with indicated antibody (table 3.1). The membrane was then washed in TBST (3x5min), incubated with indicated secondary antibody (Table 3.1) for 45 min at room temperature and washed again in TBST (3x5min). The detection was done with Clarity Western ECL substrate (Bio-Rad #170-5060), according to the manufacturer's protocol, and the membrane was scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

3.3.6 Immunoprecipitation of OVA with anti-OVA IgE (2C6)

Biotinylated anti-OVA IgE was captured with Dynabeads™ M-280 Streptavidin (Invitrogen) for 30 min at room temperature on a rotating wheel. The beads were washed with 0.1%BSA in PBS pH7.4 for five times, and the OVA-containing cell lysates or 0.1 µg OVA were added (100 µL of beads were used per sample). Antigen capturing was done for 60 min with rotation at room temperature. The beads were washed three times with 0.1% BSA in PBS pH7.4 and the antibody-antigen complexes were eluted by resuspending the beads in Laemmli Buffer with 10% β-mercaptoethanol and boiling for 5 min at 95°C. The samples were submitted to Western blot as described below.

3.3.7 NuPAGE Bis-Tris gel electrophoresis and Western blot

Samples were diluted in Laemmli Sample Buffer containing 10% β -mercaptoethanol for analysis under reducing conditions and heated at 95°C for 5 min. The samples and Precision Plus Protein All Blue Prestained Protein standard (Biorad #1610373) were loaded to pre-casted NuPAGE Bis-Tris gels 12% (Invitrogen #NP0341BOX), and the gel was run in the XCell Surelock electrophoresis system at 100V for 90 min. The semi-dry transfer was done using the Trans-Blot Turbo Mini 0.2 μ m PVDF Transfer Packs (Bio-Rad #1704156). Bio-Rad trans-blot turbo transfer system V1.02 was used. The membrane was blocked in 5% milk for one h and incubated with indicated antibodies (table). The blots were washed in TBST (3x5min), incubated with indicated secondary antibodies (table) for 45 min at room temperature and washed again in TBST (3x5min). The detection was done with Clarity Western ECL substrate (Bio-Rad #170-5060), according to the manufacturer's protocol, and the membrane was scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

Table 3.1 Antibodies and incubation conditions used in Dot Blot, Western blot and IP experiments.

experiment	Primary antibody	Secondary antibody
Dot Blot	Anti-OVA IgE (in house, clone 2C6), 10 μ g/mL, 90 min room temperature	anti-mouse IgE HRP conjugate 1: 2500 (Southern Biotech #1110-05)
WB	Anti-OVA IgE (in house, clone 2C6), 10 μ g/mL, overnight 4°C	anti-mouse IgE HRP conjugate 1: 2500 (Southern Biotech #1110-05)
	Rabbit polyclonal anti-OVA (EMD Millipore) 1:1000, 90min room temperature	anti-rabbit IgG HRP conjugate 1: 10 000
IP	Rabbit polyclonal anti-OVA (EMD Millipore) 1:1000, overnight 4°C	anti-rabbit IgG HRP conjugate 1: 10 000

3.3.8 Concentrating of cell supernatant

The cell supernatant of B16-OVA (secreted) cells was concentrated with Microsep Advance Centrifugal Device 10K (Pall corporation). By performing the centrifugation at 3000 g for 20 min, the supernatant was concentrated from 5 mL to 0.5 mL (10x).

3.3.9 ELISA

A 96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 5 μ g/mL of rabbit polyclonal anti-OVA in PBS (EMD Millipore, 100 μ L/well) overnight at 4°C. Blocking was done

with 1% BSA in PBS for one h at 37°C. Next, 50-75 µL samples or 0.08-10 µg/mL of chicken ovalbumin (Sigma #A5503) were added. The incubation was performed at room temperature for one h. Next, anti-OVA IgE (clone 2C6) was added at 1µg/mL, and the plate was incubated for one h at room temperature. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature. Immunoreactivity was visualised with TMB Stabilized Chromogen (Invitrogen). The reaction was stopped after 15 min with 0.5 M H₂SO₄, and absorbances were read at 450 nm and 620 nm.

3.3.10 Beta-hexosaminidase assay

This assay was kindly performed in Frank Redegeld's laboratory at the University of Utrecht. Bone marrow-derived mast cells (BMMC) were used. BMMC were obtained by culturing bone marrow cells from wild type mice in RPMI 1640 medium (Gibco) supplemented with 10% Fetal Bovine Serum (Wisent), 1% penicillin/streptomycin (Gibco), 1mM sodium pyruvate (Gibco), 1% Glutamax (Gibco), 20mM HEPES (Gibco), 1% MEM non-essential amino acids (Gibco), 50 µM 2-mercaptoethanol (Gibco) with the addition of 10 ng/mL of IL-3 (Prospec #cyt-371) and 10 ng/mL SCF (Prospec #cyt-275). BMMC are ready to be used from week 4 until week 8-9. For MC degranulation assay, the BMMC were primed overnight with indicated IgE antibodies at 37°C. The following day, the unbound IgE was removed by washing the cells, and activated by adding OVA-containing samples, 2 µM ionomycin (Sigma #19657) or anti-IgE antibody (BD Pharmingen #553416, 0.5 µg/mL) for 1h at 37°C. After 1h, the cell-free supernatants were harvested and mixed with 200 µM 4-methylumbelliferyl-β-D-Glucopyranoside (4-MUG) substrate in 0.1 M citrate buffer for 1 h 37°C. The reaction was stopped with 0.1 M glycine buffer. Total β-hexosaminidase content was determined by lysing the cells with 0.5 % Triton X-100. β-hexosaminidase content was quantified by fluorometric measurements at ex360nm and em460nm. The percentage of released β-hex was calculated by using the following equation: $\frac{A-B}{T-B} \times 100\%$, where A is the amount of β-hex released from stimulated cells, B is the amount of β-hex released from unstimulated cells, and T is total β-hex content.

3.3.11 OT-1 activation

OT-1 activation was performed as described in Chapter 2 (point 2.2.6).

3.3.12 *In vivo* study

C57BL/6 mice were purchased from Charles River. 50 000 B16-OVA cells (secreted OVA) were subcutaneously injected into the right flank. Antibody treatment consisted of a combination of two anti-OVA IgE antibodies (clone 2C6 (in house produced) and clone E-C1 (Chondrex #3006)), 10 µg each. The antibodies were injected on days 3, 10 and 14 intravenously. Some mice received the adoptive cell transfer of 0.25-1 million activated OT-1 cells in PBS intravenously on day 10. The tumour size was measured with a calliper, and the mice were sacrificed when the tumours reached 10 mm in diameter or at the first sign of ulceration or if significant weight loss was observed (> 20% of initial weight). Tumour volume was calculated by the modified ellipsoidal formula: $V = \frac{1}{2} (\text{Length} \times \text{Width}^2)$.

3.3.13 Histology

Histology was performed at the Easter Bush Pathology unit (University of Edinburgh). Tumours were collected at necropsy and fixed in 10% (vol/vol) phosphate-buffered formalin. Sections were cut from paraffin-embedded tissues and routinely stained with toluidine blue. The pictures of the slides were taken with the EVOS M7000 Imaging System.

3.3.14 Statistical analysis

Statistical analysis was performed in GraphPad Prism software. Survival was evaluated with the Mantel-Cox test. P-values of ≤ 0.05 were considered statistically significant. ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.4 Results

3.4.1 Validation of anti-OVA IgE antibody

Before using our in-house produced antibodies *in vivo*, a thorough characterisation was performed for each of them. In this chapter, I used the murine anti-OVA IgE antibody secreted by 2C6 hybridoma. Production, purification, and quality control of this anti-OVA IgE antibody have already been described in the published paper given in appendix¹¹³. It complied with high-quality standards, as purity and monomericity reached above 95%. However, further validation was needed regarding target antigen binding, i.e. to the version of OVA expressed in our target cell lines. Multiple assays were carried out to confirm antigen binding, as summarised in **table 3.2**. In the assays, we included a B16-F10 cell line as a negative control, chicken ovalbumin (OVA) purified from egg white as a positive control, and two B16-OVA cell lines – one expressing truncated intracellular OVA (amino acids 161-385) and one secreting the full-length OVA.

Table 3.2 Assessment of antigen-binding of anti-OVA IgE antibody via multiple techniques

Sample	Sample type	Western Blot (denaturing, 10 µg)	Dot Blot (native, low amount ~2 µg)	ELISA (native, 50-75 µL)	IP (native, high amount, ~300 µg)
B16-F10	negative control	not tested	x	x	x
B16-OVA: truncated OVA, intracellular	test	x	x	x	x
B16-OVA: full-length OVA, secreted	test	not tested	x	✓ weak signal	✓
OVA	positive control	x	✓	✓	✓

IP-immunoprecipitation; x-negative, ✓ - positive

Initially, we started with western blot analysis using the B16-OVA (i.c.) lysate and OVA protein as a control. The samples were run under denaturing, reducing conditions. The membrane was incubated with the anti-OVA IgE (2C6) as a primary antibody, followed by anti-IgE HRP conjugate. However, no bands were detected, not even for OVA positive control loaded in serial dilutions (**Fig. 3.1 A**). To exclude any potential technical problem, we incubated the same membrane with rabbit polyclonal anti-OVA antibody, followed by anti-rabbit IgG-HRP as a secondary antibody. OVA bands were detected with the polyclonal antibody, indicating that they were indeed present on the membrane, but the monoclonal IgE has not been able

to bind (**Fig. 3.1 B**). OVA from the cell lysate showed lower molecular weight when compared to OVA protein, which was expected as the tested cell line expresses truncated OVA missing 160 amino acids (**Fig. 3.1 B**). The results of western blot analysis made us suspect that anti-OVA IgE does not bind to OVA under denaturing conditions, so we decided to proceed to test under native conditions.

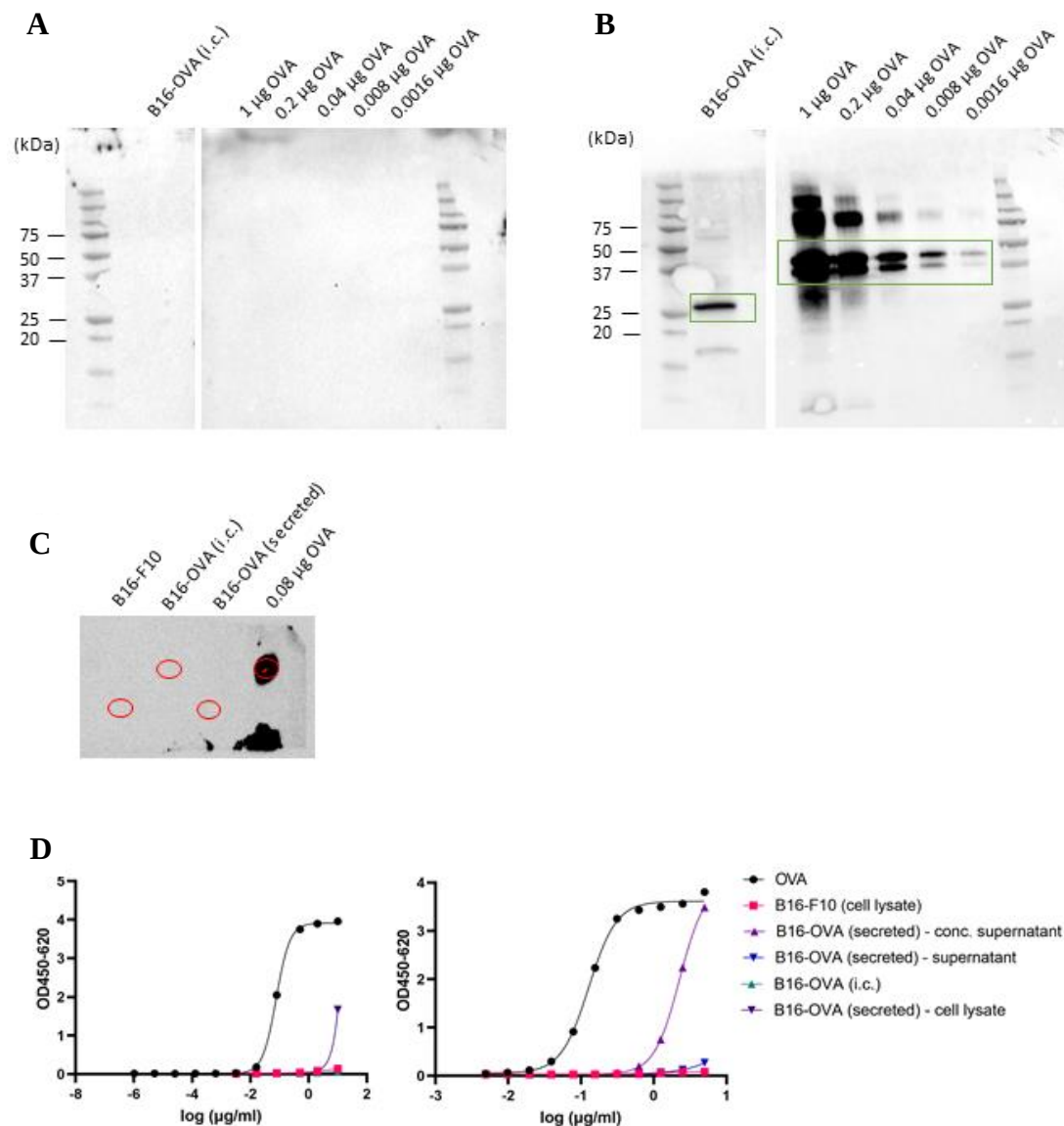


Figure 3.1. Evaluation of OVA binding capacity of anti-OVA IgE antibody (clone 2C6). Binding of 2C6 anti-OVA IgE to OVA was studied in: western blot under denaturing conditions and either (A) 2C6 anti-OVA IgE or (B) polyclonal anti-OVA antibody as a primary antibody; (C) Dot Blot with 2C6 anti-OVA, red circles indicate the spots where the samples were applied; (D) sandwich ELISA in which the Coating was done with polyclonal anti-OVA IgE. Next, the samples were added, followed by 2C6 anti-OVA IgE. The detection was done with anti-mouse IgE-HRP conjugate.)

The first technique that we decided to use was dot blot, which allows testing the antigen-antibody binding under native conditions, although no information regarding the molecular weight of the protein can be obtained. We tested all three cell lysates and OVA protein with the same combination of antibodies as used for western blot (anti-OVA IgE (2C6) + anti-IgE-HRP). Although we could not detect OVA in any lysate, OVA positive control gave a strong signal, indicating that the anti-OVA IgE can bind to OVA under native conditions (**Fig. 3.1 C**). We thought the lack of signal with our cell lysates could be due to two reasons. Firstly, the B16-OVA cell line expressing truncated i.c. OVA lacks a significant portion of OVA protein. This deleted part could contain the antibody binding site. Without it being expressed, the antibody fails to bind. Secondly, the B16-OVA cell line that secretes OVA has potentially very low amounts of OVA present in the cell lysate. In addition, the dot blot technique is limiting in terms of the sample volume that can be loaded on the membrane (a few microliters). Therefore, although, in theory, the anti-OVA IgE should be able to bind to the full-length OVA expressed in this cell line, the amount of OVA in the sample might be under the detection limit for a dot blot.

Next, to overcome the sample volume limitation, but in order to keep native conditions, we proceeded to a sandwich ELISA. Here, the coating was done with a polyclonal anti-OVA antibody in order to capture even the truncated version of OVA. Then, the samples were added, followed by anti-OVA IgE. For the B16-OVA cell line with secreted OVA, both cell lysate and supernatant (that was either concentrated or not) were tested. In this setup, anti-OVA IgE showed clear binding to OVA positive control, whereas a weak signal was observed for B16-OVA (secreted) cell lysate and non-concentrated supernatant. Concentrated supernatant, however, gave a stronger signal, indicating that the problem indeed may be the low OVA concentration in these samples (**Fig. 3.1 D**).

Finally, we decided to perform an immunoprecipitation assay to further increase the sample volume and obtain a definite proof of binding. To this end, we biotinylated the anti-OVA IgE and used streptavidin beads to capture it. Next, the samples were added (cell lysates containing ~300 µg of protein). In this way, the binding is still happening under native conditions. After the antigen capturing, the antibody-antigen complex was eluted from the beads and used in western blot under denaturing, reducing conditions. The detection was done with polyclonal anti-OVA. Therefore, if the anti-OVA IgE successfully bound OVA from

the lysates, we should detect OVA bands on a western blot. Indeed, the bands corresponding to the expected OVA size were detected for the B16-OVA cell line that secretes full-length OVA. However, no band was observed for the B16-OVA cell line with a truncated version of OVA (**Fig. 3.2**).

In conclusion, the analysis described here gave us essential information regarding the anti-OVA IgE antibody and helped us characterise its antigen binding properties. First, the anti-OVA IgE antibody (2C6 clone) only binds OVA under native conditions. Thus, it cannot be used in denaturing western blot. Secondly, as confirmed by ELISA and IP, it cannot recognise truncated OVA expressed in one of our B16-OVA cell lines of interest. Finally, the anti-OVA IgE does bind full-length secreted OVA, although it appears that the amount of OVA in tested samples was low and can be a limiting factor.

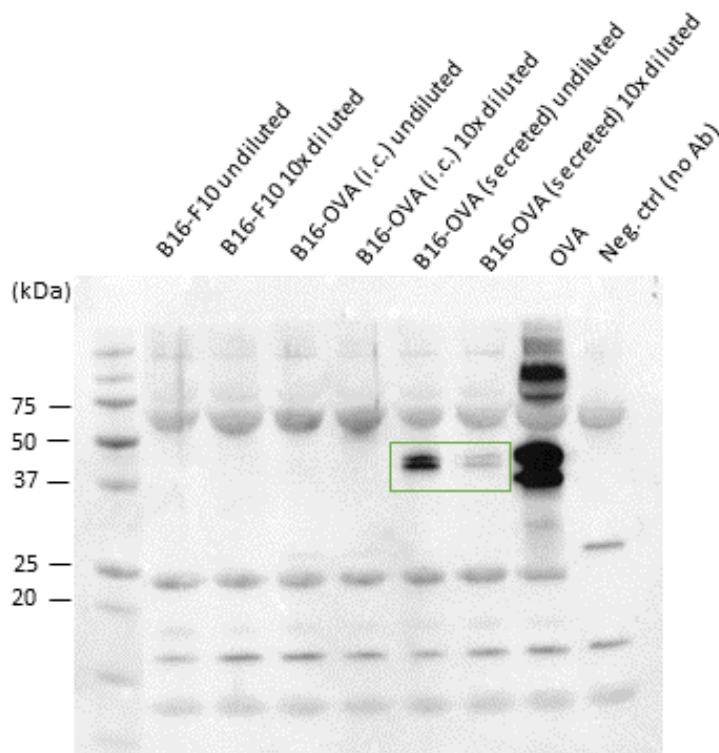


Figure 3.2. Confirmation of OVA binding in the immunoprecipitation experiment. Biotinylated anti-OVA IgE (2C6) was captured with streptavidin beads. Next, cell lysates or OVA were added for an hour, after which the antibody-antigen complexes were eluted from the beads and used for a western blot analysis with polyclonal anti-OVA antibody.

3.4.2 The combination of two anti-OVA IgE antibodies is needed to induce mast cell degranulation

The main goal of this study was to evaluate the effects that an IgE targeting a secreted tumour antigen would have on tumour growth *in vivo*. Specifically, we wanted to study the IgE-mediated MC/basophil degranulation, as MC degranulation has been previously reported to mediate tumour control⁹⁴. Therefore, we needed to test if the anti-OVA IgE antibody we intended to use *in vivo* could induce MC degranulation in response to the OVA secreted from the B16-OVA model cell line.

In collaboration with Frank's Redegeld laboratory at the University of Utrecht, we were able to study this question in an *in vitro* MC degranulation assay. In this experiment, murine bone marrow-derived MCs were sensitised with two different anti-OVA IgE antibodies (2C6 and E-C1 clones), alone or in combination. After washing the unbound IgE, the antigen-containing samples were added. The MC degranulation was quantified by measuring β -hexosaminidase release in a fluorometric enzyme assay. β -hexosaminidase is an inflammatory mediator stored in MC granules from which it is released during MC degranulation. Thus, quantification of β -hexosaminidase release is commonly used to determine MC degranulation.

Our results show that both anti-OVA IgE antibodies, when used individually, can induce MC degranulation when they encounter OVA protein, but not the secreted OVA from the B16-OVA supernatant (**Fig. 3.3**). 2C6 clone seemed to have better activity, as it induced about 40% of β -hexosaminidase release with the lowest tested OVA concentration (0.1 μ g/mL). E-C1, on the other hand, only induced 5% of release in response to the same OVA concentration. Both antibodies showed dose-dependent behaviour. Next, we tested the two anti-OVA IgE antibodies in combination in order to improve cross-linking of the Fc ϵ receptor on the MC surface. Indeed, by combining the two antibodies, MC degranulation was detected with B16-OVA cell supernatant. In addition, the percentage of β -hexosaminidase release increased to about 70% with 0.1 μ g/mL OVA protein, confirming that the combination of two IgE antibodies maximised the MC degranulation (**Fig. 3.3**).

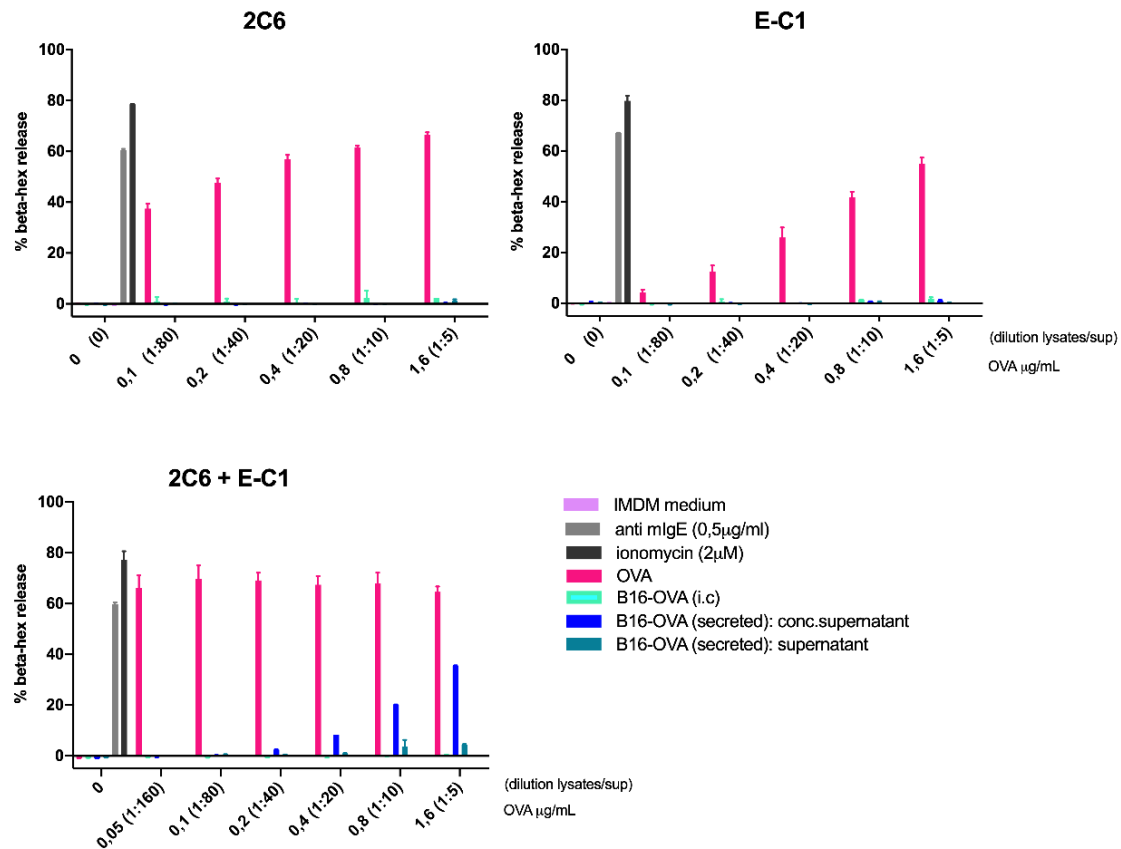


Figure 3.3. The combination of two anti-OVA IgE antibodies induces MC degranulation with B16-OVA supernatant. MC were sensitised with either 2C6 or EC-1 anti-OVA IgE, alone or in combination. OVA-containing samples were added, and the release of β -hexosaminidase was evaluated in a fluorometric assay. Alternatively, sensitised MCs were treated with ionomycin or anti-murine IgE antibody as positive controls. Mean values + SD of duplicates are shown.

3.4.3 IgEs targeting a secreted tumour antigen abolish OT-1 protective effect *in vivo*

Having characterised the anti-OVA IgE antibody and observed that the combination treatment is needed to achieve MC degranulation, we moved to the *in vivo* setting. Here, a syngeneic tumour mouse model was used in which we subcutaneously injected B16-OVA cells that secrete full-length OVA.

First, the presence of MCs in the TME of B16-OVA tumours was confirmed by histology. The tumour tissues were stained with toluidine blue, which revealed that MCs mainly localised around the tumour edges. A few MCs were also detected in the deeper tumour area (**Fig 3.4**). Of note, toluidine blue also stains basophils, so we cannot exclude the possibility that some of the observed cells are basophils.

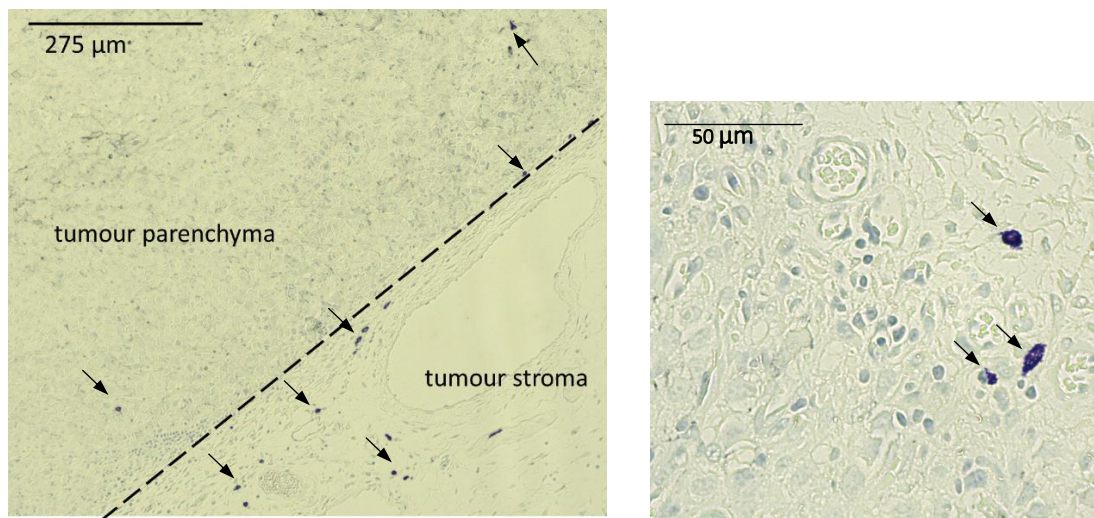


Figure 3.4. Toluidine blue staining of the B16-OVA tumour tissue reveals MC presence. Subcutaneous B16-OVA tumours from the mice that reached an endpoint were excised, fixed in formalin and embedded in paraffin. The sections were stained with toluidine blue. MCs are stained in purple (black arrows).

Next, the mice were treated as per the experimental scheme given in **figure 3.5 A**. The treatment comprised the combination of two anti-OVA IgE antibodies (2C6 and E-C1) that had been previously tested *in vitro*. In addition, the IgE treatment was combined with OT-1 adoptive cell transfer. Evaluation of the effect that IgE therapy had on CD8⁺ T cell adoptive cell therapy allowed us to detect potential immunosuppressive or immunostimulating changes in the TME.

Our results show that IgE treatment alone did not affect the tumour growth, as the tumour growth curves and median survival were not significantly different between the untreated and the IgE-treated groups (**Fig 3.5 B-D**). However, an interesting finding was made in the groups that received the OT-1 adoptive cell transfer. As expected with this B16-OVA cell line, the OT-1-treated mice developed the tumours significantly slower or not at all compared to the untreated group. However, when the IgE treatment was added, the protective effect of OT-1 therapy was entirely lost (**Fig 3.5 B-D**). The difference was also reflected in the median survival: 42 days for OT-1 treatment alone, compared to 28 days for OT-1 combined with IgE treatment (**Fig 3.5 D**). Mechanistically, we suspect that the observed loss of tumour growth control by OT-1 cells could be due to the IgE-induced changes in the tumour microenvironment.

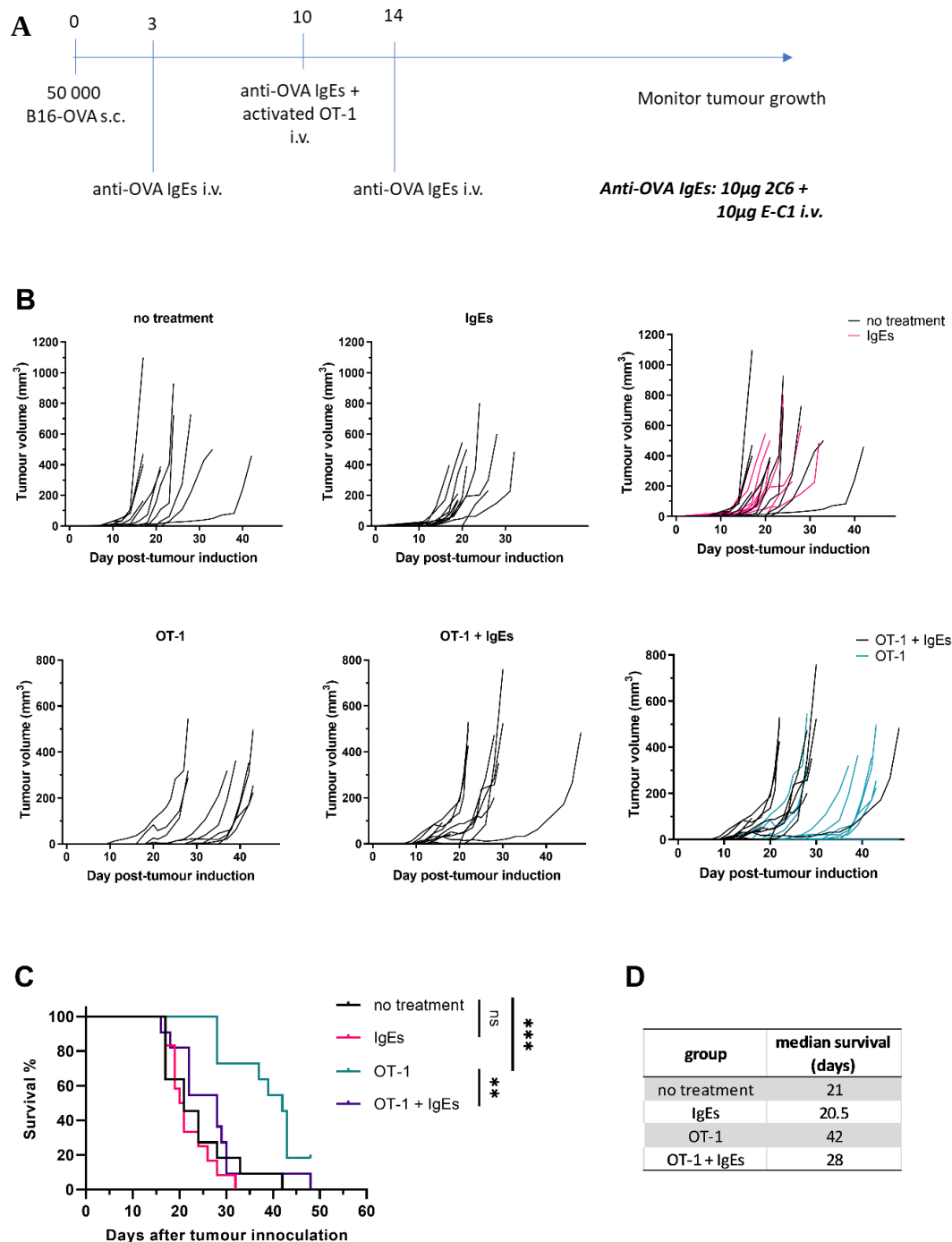


Figure 3.5. Anti-OVA IgE treatment abolishes the OT-1 protective effect in the B16-OVA model (secreted OVA) *in vivo*. C57BL/6 mice were subcutaneously injected with 50 000 B16-OVA cells which secrete OVA in the flank. They were treated with the combination of two anti-OVA IgE antibodies (clones 2C6 and E-C1) with or without OT-1 adoptive cell transfer. (A) Experimental scheme. (B) Tumour growth curves are shown for each treatment group alone (left and middle) or overlapped (right). (C) Survival analysis. (D) Median survival in days. Combined data of two independent experiments are shown (n=11-12). Statistical significance was calculated with the Mantel-Cox test. ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.5 Discussion

MCs and basophils are known to have both pro-tumorigenic and anti-tumorigenic roles, depending on the tumour context. Thus, IgE-mediated activation of these effector cell types could also lead to different outcomes in cancer. Here, I showed that targeting a secreted tumour antigen with IgE antibodies has a detrimental effect, as it abolishes the protective effect of CD8⁺ T cell adoptive transfer.

This finding strongly suggests that IgE treatment reshaped the TME in an unfavourable way. Since the secreted antigen is constantly released from the tumour cells, we believe that such a constant presence in the TME may have induced a similar type of chronic activation described at the allergy sites. This includes the release of immunosuppressive molecules, such as TGF- β , IL-10 and adenosine, as well as the support of MDSC and T_{reg} populations⁸⁰. Furthermore, chronic skin inflammation has been reported to promote carcinogenesis by raising the polyclonal IgE levels and subsequent basophil recruitment and activation³⁸. In addition, histamine signalling through the H2 receptor can inhibit Th1 and Th2 responses¹²⁶, further contributing to the immune-suppressive environment. Finally, MCs can recruit neutrophils^{80,127}, whose immunosuppressive roles in cancer are well-established¹²⁸.

However, further studies are needed to elucidate the underlying mechanisms of the observed effect of IgE treatment. The first step would be to confirm that the loss of OT-1 efficacy is a consequence of MC and basophil activation. For this purpose, the same experimental setup can be used in MC-deficient¹²⁹ and basophil-deficient mice¹³⁰ to confirm which cell population is critically involved. Furthermore, to evaluate if degranulation of effector cells is needed, mice could be treated with only one anti-OVA IgE antibody instead of two. *In vitro*, neither 2C6 nor E-C1 anti-OVA IgE antibody was sufficient to induce MC degranulation; thus, we expect the single-agent treatment to behave similarly *in vivo*. Alternatively, histamine inhibitors or MC stabilisers could be included in the treatment regimen.

Once we establish the cell population that mediates the loss of ACT efficacy and if degranulation is necessary for such an effect, it would be interesting to study the changes in the TME cell composition. The presence of MDSC, M2 macrophages, T_{reg}, as well as CD8⁺/T_{reg} ratio, could be determined by flow cytometry to evaluate the immunosuppressive state

potentially induced by IgE treatment. In addition, immunosuppression could definitely be confirmed as the underlying mechanism by adding the anti-PD-L1 or anti-CTLA-4 antibodies to the *in vivo* treatment scheme. In such a case, the checkpoint inhibitors would be expected to rescue the OT-1 efficacy by reverting the IgE-induced immunosuppressive TME. Taken together, this set of experiments would help us decipher the exact mechanism by which IgE treatment targeting a secreted tumour antigen abolished the protective effect of CD8⁺ T cell ACT.

Limitations

Although the IgE-induced MC degranulation was confirmed *in vitro*, the *in vivo* evaluation of MC degranulation in the tumour can be challenging. It would require the analysis of the tumour material shortly after IgE injection either by histology (staining of MCs with toluidine blue) or, alternatively, the activation status of MCs could be evaluated by quantifying different cytokines and chemokines in the tumour supernatants by ELISA⁹⁰. Another possibility is the intravenous injection of Evans Blue (EB) and measurement of local EB extravasation at the tumour site¹³¹. MC degranulation would make the tissue more permeable, and extravasated EB would colour it blue. However, in every of the mentioned approaches, the critical point would be to find the correct timing, as it is difficult to predict how fast IgEs activate the MCs at the tumour site. Thus, measuring local MC activation is not straightforward, but it should be considered in future experiments.

Furthermore, using such highly immunogenic antigen as OVA as a model tumour antigen in this study may not be fully translatable to human settings where tumour antigens are often weak immunogens. Thus, the findings described in this chapter should be confirmed with a secreted tumour antigen that is less immunogenic. For example a human transgenic PSA mouse can be transplanted with a tumour cell line expressing human PSA and treated with anti-PSA IgE antibodies as a model system¹³²⁰.

Conclusions

In summary, in this chapter, I have first shown that anti-OVA IgE (2C6 clone) only binds native OVA and is unsuitable for OVA detection under denaturing conditions. Next, the combination of two anti-OVA IgEs was needed to induce MC degranulation against the OVA secreted from B16-OVA cells. Finally, targeting a secreted antigen with IgE antibodies abolished the

protective effect of CD8⁺ T cell ACT in mice. These results strongly indicate that IgE against a secreted antigen can induce harmful changes in the TME, ultimately leading to worse CD8-mediated tumour control. A set of different experiments was suggested to help elucidate the underlying mechanism of the observed effect.

4 Chapter 4: Targeting an intracellular tumour antigen by immunising the mice under type-2 immune skewing conditions

4.1 Abstract

In this chapter, I continue exploring different antigen types as potential IgE targets. Here, intracellular tumour antigen was chosen to achieve an acute antigen release from tumour cells, thus avoiding chronic activation of IgE-effector cells at the tumour site, which we suspect had detrimental effects in chapter 3. To this end, I used two in vivo models: B16-OVA and the spontaneous prostate cancer model (TRAMP). Due to the unavailability of IgE antibodies against potential intracellular targets in these models, we decided to raise polyclonal IgE response via immunisation under type-2 immune skewing conditions. The mice also received the CD8⁺ T cell adoptive transfer in both models. My results indicate that immunisation raised both IgE and IgG responses, which, combined with ACT, led to better animal outcomes in both models. This suggests that immunisation induced favourable changes in the TME. Further work is needed to elucidate the underlying mechanism. However, we hypothesise that the IgE-MC-histamine-T_{reg} axis might be responsible for the observed effect, as represented in the figure below.

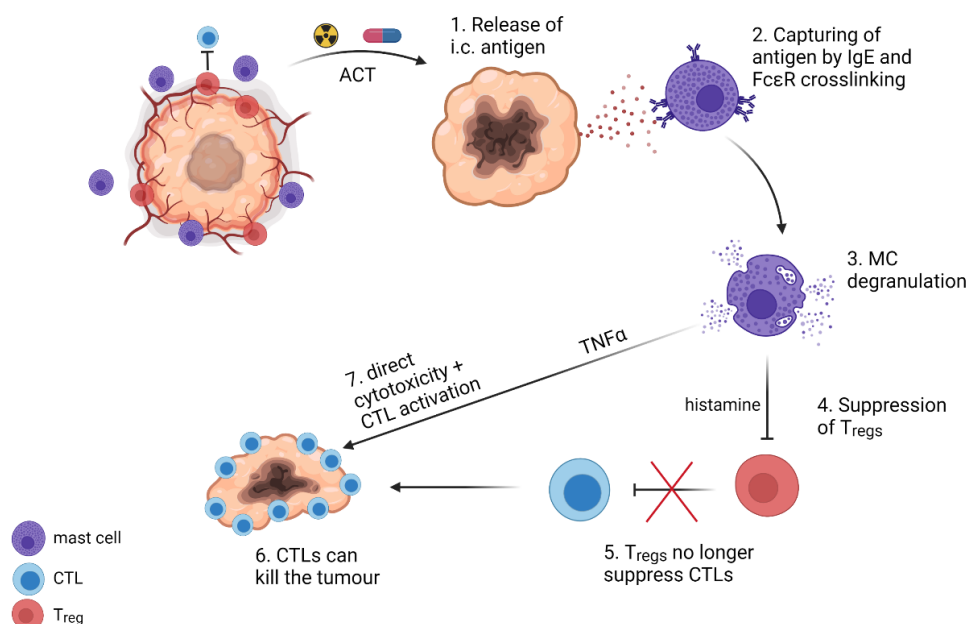


Figure 4.1. Proposed mechanism of IgE-induced tumour killing via MCs. IgE targeting an intracellular tumour antigen is captured by its high-affinity FcεRI on MCs. When intracellular tumour antigen is released from a dying tumour cell (this can be induced through radiation, chemotherapy, or ACT), it binds to IgE-coated MCs and induces MC degranulation. MCs degranulate histamine, which suppresses T_{reg} function, as well as cytotoxic TNFα, ultimately resulting in enhanced tumour killing by cytotoxic T lymphocytes (CTLs).

4.2 Introduction

It has been established that MCs affect tumour development both positively and negatively via multiple mechanisms. MC degranulation is a particularly interesting, but unexplored, way of achieving the anti-tumour effects that includes the release of cytotoxic TNF α and other mediators, such as histamine. MC-derived histamine has been shown to enhance the Th1 response through H1R signalling¹²⁶. In addition, it can also suppress T_{reg} function⁹¹ and indirectly enhance NK cell activity. By signalling through H2R on monocytes and phagocytes, histamine prevents ROS-mediated downregulation of NKp46 and NKG2D stimulatory receptors on NK cells¹³³. Further supporting the anti-tumour role of histamine is its success in enhancing the efficacy of IL-2 immunotherapy in clinical settings^{134,135}. In addition, it has been shown that histamine deficient mice are more susceptible to carcinogen-induced skin and colorectal cancer due to impaired myeloid cell differentiation¹³⁶.

As discussed in chapter 1, MC degranulation has been reported to mediate tumour growth control⁹⁴ and break peripheral tolerance in the skin graft model by suppressing T_{reg} activity⁹⁰. Thus, we believe that IgE-induced MC degranulation can be exploited as an anti-tumour approach, with a particular interest in histamine. However, the data presented in chapters 2 and 3 of this thesis indicate that antigen localisation plays an important role in the outcome of IgE-mediated activation of MCs (and potentially basophils). For instance, targeting a surface tumour antigen did not induce any change in the tumour growth, whereas targeting a secreted antigen was detrimental in terms of abolishing the T cell protective effect. Here, we decided to target an intracellular antigen to avoid the constant presence of the antigen in TME and chronic activation of IgE effector cells, which seemed to have induced the loss of CD8⁺ T cell-mediated tumour control in chapter 3. We hypothesise that occasional release of the intracellular antigen leads to acute IgE-mediated effector cell degranulation. The release of TNF α and histamine can then potentially mediate tumour clearance.

Initially, we wanted to explore this idea in the B16-OVA model by using the B16-OVA cell line that expresses truncated OVA intracellularly and the anti-OVA IgE antibody. However, my data show that the anti-OVA IgE that we had available does not bind to the OVA present in this cell line (chapter 3, section 3.3.1). In addition, even with full-length OVA, the combination of two anti-OVA IgE antibodies was necessary to induce MC degranulation (chapter 3, section

3.3.2). Alternatively, intratumoral MC degranulation can be induced by a compound named 48/80¹³⁷. However, such intratumoral injection in our hands led to many ulcerating tumours, leading to premature experiment termination and complex evaluation. Thus, we took a different approach consisting of induction of Th2 response via immunisation to raise a polyclonal IgE response against an intracellular antigen of choice. Such an IgE polyclonal response would be sufficient to induce effector cell degranulation successfully.

To this end, we used the B16-OVA syngeneic model (expressing intracellular OVA) and TRAMP (transgenic adenocarcinoma mouse prostate) model of spontaneous prostate cancer. OVA and SV40 large T antigen (SV40-Tag) were chosen as target intracellular tumour antigens, respectively. In both cases, the ACT of activated CD8⁺ T cells was included to induce the release of the intracellular antigen from the tumour cells. As we hypothesise that IgE-mediated histamine release from MCs can lead to tumour rejection via T_{reg} suppression, the adoptive T cell transfer also allowed us to evaluate the T_{reg} function. In both chosen models, T_{regs} are known to play an essential role in tumorigenesis. For example, the used B16-OVA tumour cell line is generally resistant to adoptive T cell transfer monotherapy¹¹⁴ (also not published observations from our group). However, depletion of tumour-residential T_{regs} significantly enhances the rejection of these tumours in the context of anti-tumour immunisation¹¹⁸. Thus, the efficacy by which transferred T cells reject B16-OVA tumours can be correlated with the functionality of tumour-intrinsic T_{regs}. As for the TRAMP model, it has been reported that the application of CTLA-4 checkpoint inhibitors diminishes tumour growth¹³⁸. In addition, although the transfer of antigen-specific CD8⁺ T cells leads to a significant delay in tumour development when mice are still young¹³⁹, it has no effect when neoplastic lesions are fully developed¹⁴⁰. Notably, immunosuppressive TGFβ plays a crucial role in limiting the T cell efficacy in older mice¹⁴⁰. These findings suggest that T_{regs} and an immune-suppressive environment protect the tumour from CD8⁺ T cell-mediated killing. Therefore, the TRAMP model also seems suitable to test our hypothesis regarding histamine-induced T_{reg} suppression.

My results indicate that immunising the mice under type-2 immune skewing conditions against an intracellular tumour antigen in combination with ACT achieves superior tumour control than immunisation or ACT alone. Thus, it appears that the immunisation led to a successful T_{reg} suppression in the TME, ultimately allowing the adoptively transferred CD8⁺ T

cells to exert their anti-tumorigenic function. This was observed in both syngeneic and spontaneous tumour models, making us confident that this mechanism should be exploited further.

4.3 Materials and methods

4.3.1 Cell culture

B16-OVA cells were kindly gifted by Ton Schumacher (The Netherlands Cancer Institute). They were cultured in IMDM medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco) and 50 μ M 2-mercaptoethanol (Gibco).

Plat-E cells were kindly gifted by Rose Zamoyska (University of Edinburgh), and they were cultured in IMDM medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 50 μ M 2-mercaptoethanol (Gibco), 10 μ g/mL blasticidin and 1 μ g/mL puromycin. Culture conditions for B16-OVA cells and Plat-E cells were maintained at 37°C in a humidified atmosphere containing 7% CO₂.

Sf9 insect cells (*Spodoptera frugiperda* female ovarian cell line, ATCC CRL-1711) were used for the production of baculovirus and protein production. They were grown in suspension in Insect-XPRESS Protein-free Insect Cell Medium (Lonza, BE12-730Q) supplemented with L-glutamine (1%), 50 U/mL penicillin, and 50 μ g/mL streptomycin at 27°C with 135 rpm shaking.

4.3.2 Mice

C57BL/6 mice were purchased from Charles River. TRAMP mice were purchased from Jackson Laboratory. The correct genotype of TRAMP mice was confirmed by PCR on DNA extracted from ear notches using

5'-CCGGTCGACCGGAAGCTTCCACAAGTGCATTTA-3' forward primer and

5'-CTCCTTTCAAGACCTAGAAGGTCCA-3' reverse primer.

4.3.3 B16-OVA *in vivo* model

C57BL/6 mice were immunised twice with 10 μ g OVA (Sigma-Aldrich #A5503) in 1:1 ratio PBS: Alum (ThermoScientific #77161) on day 0 and day 14 of the experiment. On days 21-25, the mice were nebulised with 2.5% (w/v) OVA in PBS for 20 min. The nebulisation was then repeated once weekly for three more weeks. On day 28, the mice were subcutaneously injected with 20 000 B16-OVA cells in PBS in the right flank. The mice were then randomised

within non-immunised and immunised groups. Ten days later (day 38), the adoptive cell transfer of 250 000 activated OT-1 cells in PBS was done intravenously. The tumour size was measured with a calliper, and the mice were sacrificed when the tumours reached 10 mm in diameter or at the first sign of ulceration. The weight was monitored regularly, and the maximum loss of 20% of the initial weight was allowed. Tumour volume was calculated by the modified ellipsoidal formula: $V = \frac{1}{2} (\text{Length} \times \text{Width}^2)$.

4.3.4 OT-1 activation

OT-1 activation was performed as described in Chapter 2 (section 2.2.6).

4.3.5 Cloning of SV40 large T

pSG5 Plasmid encoding for SV40 large T antigen was bought from Addgene (#9053). SV40 large T antigen sequence was cloned into pFastBac™1 expression vector using standard cloning techniques. In short, the plasmids were digested with BamHI and run on a 1% agarose gel for 1h at 120V. The expected bands were excised from the gel and extracted with the Qiagen Gel Extraction Kit. Next, the Rapid DNA Dephos & Ligation Kit (Roche) was used for vector dephosphorylation, followed by ligation reaction in 1:3 molar ratio vector: insert. DH5α competent cells were transformed with the ligation reaction and plated on LBamp plates. The following day, colonies were picked, and miniprep was done. The screening of the clones was done with HpaI restriction, and the correct clone was confirmed by Sanger sequencing (Genewiz).

4.3.6 SV40 large T production

SV40 large T was produced using a Baculovirus expression system. First, pFastBac™1_SV40 Large T plasmid was transformed into DH10 Bac cells (Gibco 10361012). During this process, the transposition takes place and deletes the LacZ gene present in BacY cells. Therefore, the colonies that contain newly formed bacmid with the gene of interest are white, whereas the blue colonies indicate that no transposition occurred. The transformation mix was spread onto agar plates containing: 1mM IPPG, 100ug/mL X-Gal, 50μg/mL kanamycin, 7μg/mL gentamycin and 10μg/mL tetracycline. After two days, a few white colonies were picked, and miniPrep was done. To confirm the transposition of the insert, the PCR with M13 primers was done on bacmid DNA. Next, Sf9 cells were transfected with the bacmid DNA to generate P0 viral stock as follows. 15 μL FuGENE® HD (Promega) and 20 μL bacmid DNA were each mixed

with 600 μ L Lonza Insect Xpress medium. Then they were mixed together and incubated for 20 minutes at room temperature. 200 μ L transfection mixture was then added dropwise per well of a 6-well plate containing 9×10^5 Sf9 cells in a 2 ml antibiotic-free medium (80% confluent). The cells were incubated at 27°C, and after 5 days, the supernatant containing P0 baculovirus was collected and 0.22 μ m filtered. Next, 1 mL of the P0 virus was added to 50 mL of Sf9 cells at the density of 2 million/mL. After two days of shaking at 135rpm at 27°C, the culture was centrifuged at 2000g for 5 minutes, the supernatant with P1 virus was filtered and stored at 4°C. Next, 600 mL of Sf9 cells at the density of 3 million/mL were infected with 10 mL of the P1 virus. After two days, the cells were pelleted, and the pellet was resuspended in 30 mL of the Tris-based lysis buffer containing 1% TritonX and 0,25mM AEBSF (protease inhibitor). The lysis was completed with sonication on ice, after which the sample was pelleted again in a table centrifuge at maximum speed for 10 minutes at 12°C, and the supernatant was submitted to purification.

4.3.7 SV40 large T protein purification

The purification was done in two steps, anionic exchange (AE) followed by size-exclusion chromatography (SEC). The elution fractions were tested for SV40 presence by western blot at various stages. The fractions of interest were combined together in the final sample, which was sterile-filtered and frozen at -80°C in 10% glycerol.

4.3.8 SDS-PAGE and Western blot

Samples were diluted in Laemmli Sample Buffer containing 10% β -mercaptoethanol for analysis under reducing conditions and heated at 95°C for 5 min. The samples and EZ-Run™ Prestained Rec Protein Ladder (Fisher BioReagents) were loaded to 8% polyacrylamide gel, and the gel was run at 70V for 30 min, after which the voltage was increased to 120V for 60 min. Gels were then either submitted to Coomassie blue staining or western blotting. The staining was done using GelCode™ Blue Safe Protein Stain (Thermo Scientific) for 30-40min, after which the gels were fixed for 35min in buffer containing 40% ethanol and 10% acetic acid, destained with distilled water and scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

For western blot, semi-dry transfer was done at 200 mA for 60 minutes in Owl™ HEP1 electroblotter (ThermoScientific). The membrane was blocked in 5% milk for 1h, incubated

with anti-SV40 rabbit antibody 1:1000 (Cell Signaling Technology #15729) at 4°C overnight, washed in PBST (3x5min), incubated with swine anti-rabbit-HRP secondary antibody 1:2000 (Dako P0217) for 45min at room temperature and washed again in PBST (3x5min). The detection was done with ChemiGlow West Chemiluminescence Substrate Kit (ProteinSimple), and the membrane was scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

4.3.9 T cell transduction

pMX-SV40_{IV-Cys}- β -P2A-SV40_{IV-Cys}- α vector encoding for SV40 TCR α and TCR β chains was kindly gifted by Ton Schumacher (The Netherlands Cancer Institute). For the generation of retroviruses, Plat-E cells were used. To achieve ~80% confluency on the day of transfection, 600 000 Plat-E cells were seeded in a 6cm dish on a previous day. The transfection was done with FuGENE® HD (Promega). In short, 10 μ L FuGENE was diluted in 150 μ L OptiMEM, whereas 1.5 μ g pCI-eco-packaging plasmid and 2.6 μ g pMX_SV40TCR plasmid were diluted in 50 μ L OptiMEM. DNA was added to FuGENE and incubated at room temperature for 15 min, and 200 μ L of the transfection mix was added dropwise to Plat-E cells. The following day the medium was replaced, and the 24-well non-tissue culture treated plates were coated with 20 μ g/mL retronectin, 750 μ L/well, at 4°C overnight. The next day, the supernatant of Plat-E cells containing retroviruses was centrifuged at 300g for 5min. Retronectin was removed from the plate, the wells were blocked with 500 μ L sterile 2% BSA for 30min, and the wells were washed twice with 1 mL PBS. 1 million of T cells (50 μ L of cells at 20million/mL) that were obtained by activating freshly isolated splenocytes (from non-transgenic littermate) for 48h with 5mg/mL concanavalin A and 10 μ g/mL hIL-7 was added per well together with 0.5mL of the virus supernatant. The plate was centrifuged for 90min at 500g at 32°C, without a brake. Finally, the medium was removed, and fresh IMDM was added (1 mL/well). 24h after spinfection, T cells were prepared for injections by washing in 2% FBS and resuspending in PBS. Part of the transduced T cells was saved for FACS analysis and stained with anti-CD8 PerCP Cy5.5 (Biolegend #100734) 1:50, and anti-V β 9-PE (Biolegend #139803) 1:350 at 4°C for 30 min. Samples were analysed by FACS Canto™ II (BD) using the software program BD FACSDiva.

4.3.10 SV40 immunisation protocols

TRAMP mice were immunised twice with 8 µg SV40 large T and 2 µg house dust mite extract (Citeq Biologics 02.01.85) in 1:1 ratio PBS: Alum (ThermoScientific #77161) at the age of 10.5 and 12.5 weeks. One week after the second i.p. injection, the mice were intranasally challenged for five consecutive days with 50 µg HDM and 9 µg SV40 large T in PBS.

4.3.11 TRAMP adoptive cell transfer (ACT)

About 22h before ACT, 15.5-week old male TRAMP mice were submitted to 5Gy total body irradiation to induce host conditioning. Each mouse received about 2×10^5 SV40_{IV}-TCR-transduced CD8⁺ T cells intravenously. The mice were sacrificed ~four weeks after ACT, at the age of 20 weeks.

4.3.12 Serum isolation from blood and preparation for ELISA

Once the mice were sacrificed, the whole blood was obtained by decapitating the head and draining the blood from the neck veins. The blood was incubated in untreated tubes at room temperature for 30-60min. The samples were centrifuged at 10 000g for 10min at 4°C. The top layer corresponding to serum was collected, and it was either used immediately or frozen at -80°C for long term storage. For quantifying anti-SV40 and anti-OVA IgG levels and total IgE levels, serum was used untreated, whereas the IgG-depleted serum was used for quantifying anti-SV40 and anti-OVA IgE levels. In brief, serum was incubated with protein G resin (Abcam #ab193259) in 1:1:1 serum: protein G: PBS volumetric ratio at 4°C overnight on a rotating wheel. The following day, the samples were centrifuged for 3 min at 100g at 4°C, and IgG-depleted serum was collected.

4.3.13 Serum ELISA

For quantifying anti-OVA IgG and IgE antibodies, 96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 5 µg/mL of OVA (Sigma-Aldrich #A5503) in PBS (100 µL/well) overnight at 4°C. For quantifying anti-SV40 IgG and IgE antibodies, the coating was done with 5 µg/mL of SV40 large T protein in PBS (produced as described above, 100 µL/well). Blocking was done with 1% BSA in PBS for 1 h at 37°C. Next, untreated or IgG-depleted serum was added to the plates in 5x serial dilutions. Also, the following control antibodies were used starting at 10 µg/mL: anti-OVA IgG2a (in house produced), anti-OVA IgE (in house produced),

IgE isotype (Biolegend #401701), anti-SV40 IgG2 (BD Biosciences #554149) and IgG2 isotype (eBioscience #16-4724-85). The incubation was performed at 37°C for 1 h. Finally, goat anti-mouse IgG-HRP conjugate (Southern Biotech 1:5000) or goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000) in 1:1 1% BSA PBS/PBST were added, respectively, for 45 min at room temperature.

For quantifying total IgE serum levels in both OVA-immunised and SV40-immunised mice, 96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 0.15µg/mL of goat anti-mouse IgE in PBS (Southern Biotech, 100µL/well) overnight at 4°C. Blocking was done with 1% BSA in PBS for 1 h at 37°C. Next, the mouse serum was added in 5x serial dilutions. Anti-OVA IgE (in house produced¹¹³) and IgG2 isotype (eBioscience #16-4724-85) were used as controls. The incubation was performed at 37°C for 1 h. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature.

For all ELISAs, the immunoreactivity was visualised with TMB substrate (Biolegend #421101). The reaction was stopped after 15 min with 0.5 M H₂SO₄, and absorbances were read at 450 nm and 570 nm.

4.3.14 Histopathology

Histopathology was performed at the Easter Bush Pathology unit (University of Edinburgh). B16-OVA tumours, and prostates and seminal vesicles of TRAMP mice were collected at necropsy and fixed in 10% (vol/vol) phosphate-buffered formalin. Sections were cut from paraffin-embedded tissues and routinely stained with hematoxylin and eosin. Pathological examination and classification of the prostate lobes were performed blindly by a veterinary pathologist.

4.3.15 Statistics

Statistical analysis was performed in GraphPad Prism software. P-values of ≤0.05 were considered statistically significant.

4.4 Results

4.4.1 OVA immunised mice raise polyclonal anti-OVA response

Acute allergen challenge with OVA is a commonly used asthma model in mice, characterised by elevated IgE levels, airway inflammation and increased eosinophil count in BAL and lung tissue¹⁴¹. Here, we took advantage of this model as the primary goal was to induce a strong IgE response against OVA, our model intracellular tumour antigen in B16-OVA cells. Wild type C57BL/6 mice were immunised following the treatment scheme in **figure 4.2**. The immunisation consisted of two intraperitoneal injections of OVA protein and Alum, which was used as an adjuvant known to induce type 2 response, followed by inhalation of nebulised OVA on five consecutive days. Inhalation of OVA was then repeated once weekly.

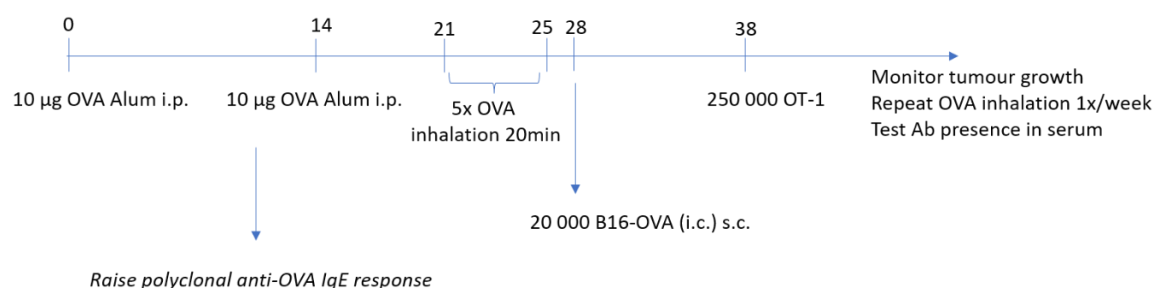


Figure 4.2. *In vivo* experimental scheme for type-2 immune skewing in B16-OVA model. The mice were immunised according to the indicated protocol, after which B16-OVA cells expressing intracellular OVA were subcutaneously injected. Ten days after the tumour cell implantation, the mice received the ACT of activated OT-1 cells i.v.

Raised antibody levels in mouse serum were studied by ELISA for anti-OVA IgG, anti-OVA IgE and total IgE serum levels. We detected anti-OVA IgG and IgE antibodies in the serum of the immunised mice, with IgG levels being significantly higher (**Fig. 4.3 A-B**). This is expected as IgE has a notably lower serum half-life than IgG (few days for IgE vs few weeks IgG). In addition, the detection of antigen-specific IgE is technically more complicated. If the plate coating is done with the antigen (OVA), it requires the removal of IgG first. IgG is present in significantly higher concentrations in the serum and would outcompete the IgE. Thus, before antigen-specific IgE ELISA, the IgG should be removed by incubating the serum with protein A or G.

Alternatively, total IgE serum levels can be used as a surrogate measurement for antigen-specific IgE levels. For example, a strong correlation between total IgE and OVA-specific IgE has been reported in OVA-immunised mice¹⁴². Therefore, we also quantified the total IgE levels in a sandwich ELISA with an anti-IgE antibody. Total IgE levels were significantly increased in the serum of immunised mice, confirming that IgE response was successfully induced (**Fig. 4.3 C-D**). Taken together, these experiments show that the OVA immunisation protocol induces a polyclonal anti-OVA response that includes both anti-OVA IgG and anti-OVA IgE antibodies.

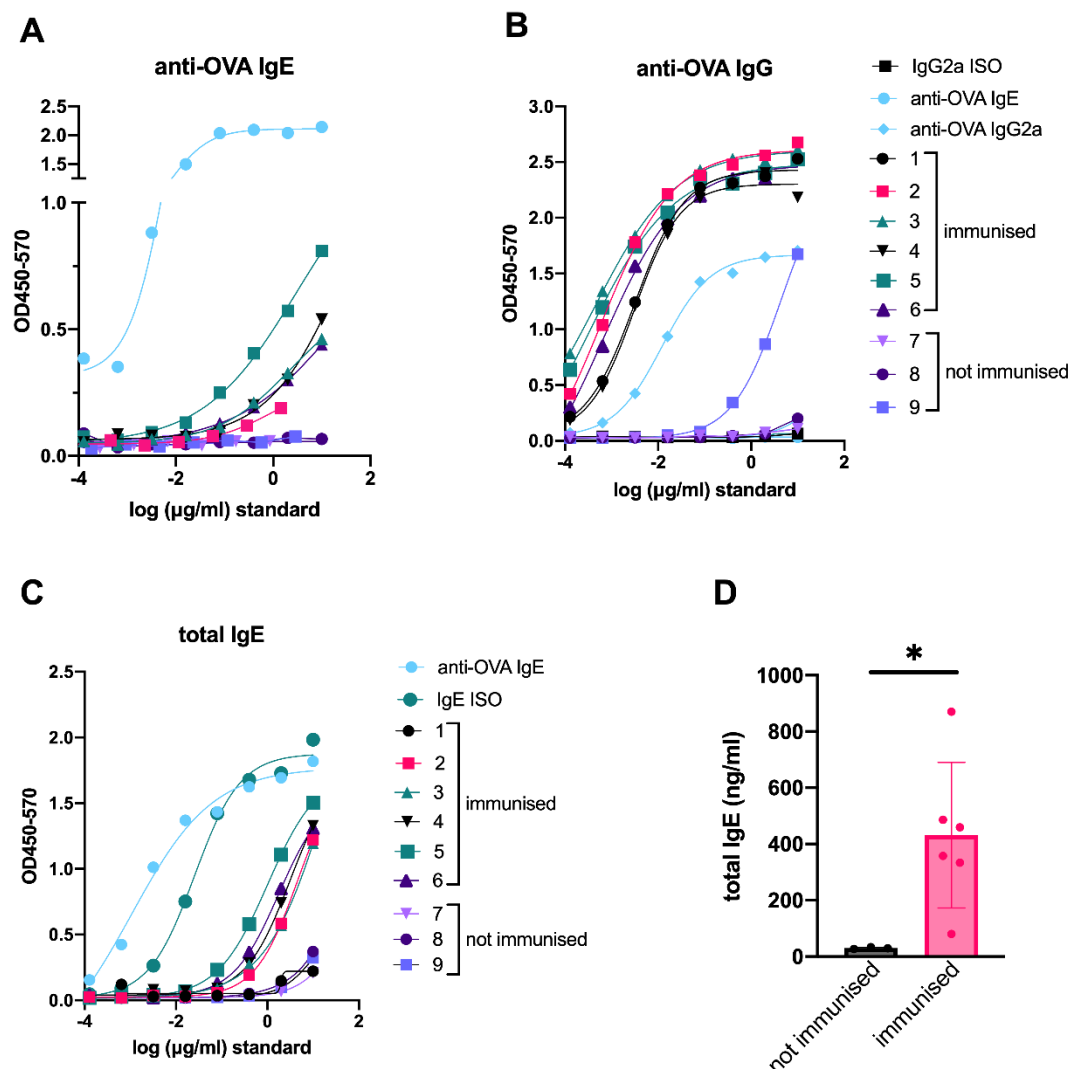


Figure 4.3. OVA immunisation protocol raises polyclonal anti-OVA response in mice. The mice were immunised according to the protocol shown in the figure 4.2. The serum was collected at the end of experiment when the animals reached the endpoint in terms of tumour size or ulceration. ELISA was used to measure the levels of (A) anti-OVA IgE, (B) anti-OVA IgG and (C) total IgE in mouse serum. (D) Quantification of total IgE levels in the serum based on the standard curve of a control IgE (IgE ISO). The statistical significance was calculated with a Mann-Whitney test. The experiment was done once.

4.4.2 Type-2 immune skewing in combination with OT-1 adoptive cell therapy seem to control the tumour growth in a syngeneic tumour model

Next, in order to study the effects that immunisation may have on tumour growth, we subcutaneously injected B16-OVA cells that express intracellular OVA into the flank of OVA immunised mice. In addition, the mice received adoptive cell transfer of activated OT-1 cells ten days after the tumour cells were injected. The OT-1 treatment was included with the same goal as in chapter 3 when we were targeting a secreted antigen - to allow for evaluation of potential immune-suppressive or stimulatory changes in the TME. Our results show that the immunised mice that received OT-1 transfer had delayed tumour development compared to other groups (**Fig. 4.4 A**). At the end of the experiment, four out of five mice from the treatment group were still alive with very small tumours or no tumour at all (**Fig. 4.4 B**). On the other hand, in the control groups, the survival was as follows: 1/3 mice in the untreated group, 1/3 in the OT-1 treated group and 1/5 in the group that was immunised but did not receive OT-1 transfer (**Fig. 4.4 B**). Unfortunately, the experiment had to be ended prematurely before the complete survival evaluation could be made. The longer follow-up would have been particularly important for the immunised mice that received OT-1 transfer, as 80% were still alive and far from an endpoint.

Nevertheless, we were able to carry out a histological analysis of the tumours. H&E staining revealed prominent lymphocyte infiltration in the group that was immunised and received ACT compared to all other groups (**Supp. Fig. 1**). These findings might indicate that T_{reg} s were indeed suppressed, allowing for major CTL infiltration. However, an analysis including lymphocyte quantification performed by a trained pathologist and larger sample number would be essential to confirm this observation.

Taken together, there are indications that the immunisation-induced polyclonal response against OVA has a synergic effect with OT-1 adoptive cell transfer in controlling the tumour growth of B16-OVA cells *in vivo*. However, since the animal groups were small (n=3-5) and the *in vivo* experiment was done only once, no firm conclusion can be made at this point.

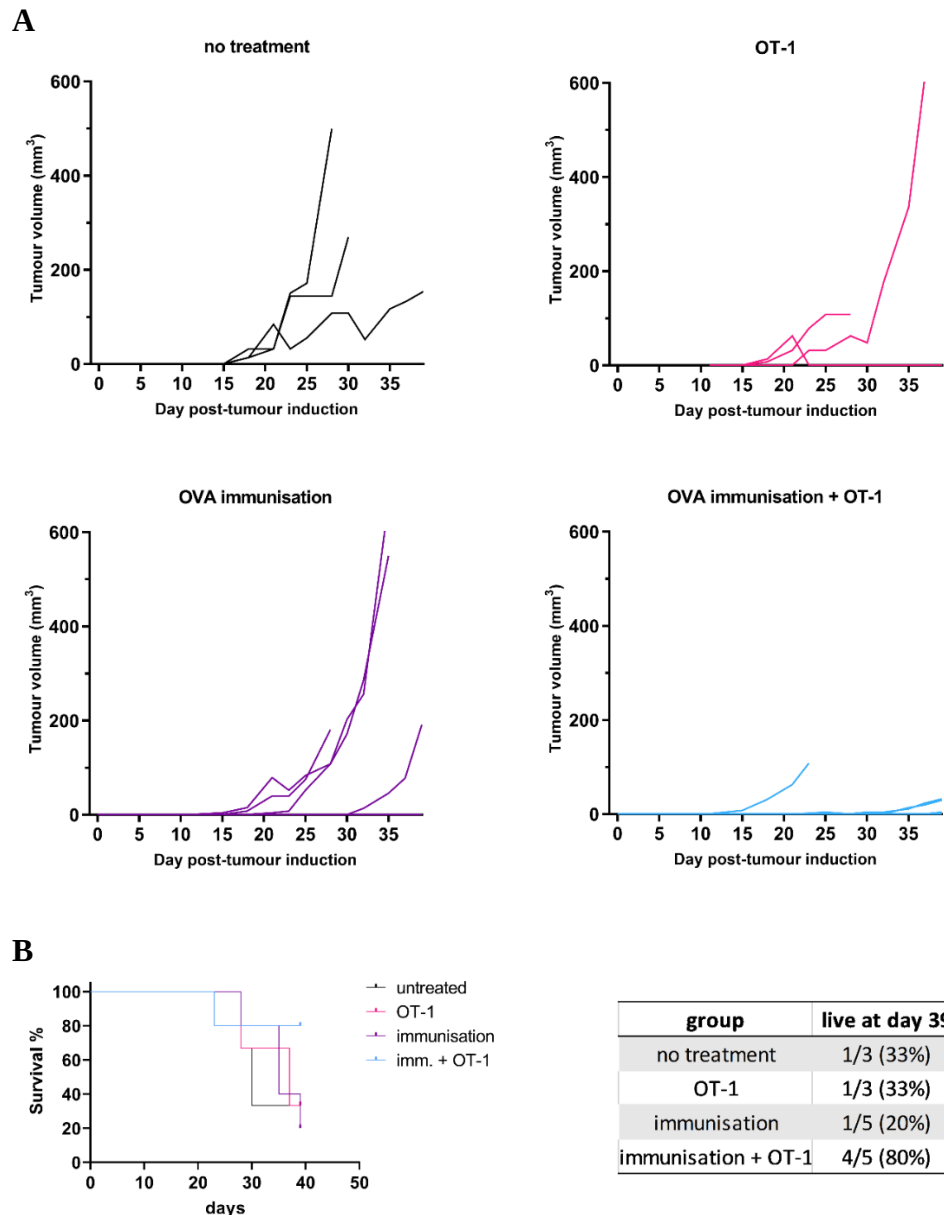


Figure 4.4. OVA immunisation and OT-1 ACT exhibit superior tumour control *in vivo*. The mice were treated as per the experimental scheme given in figure 4.2. (A) Tumour growth curves. (B) Survival analysis represented graphically (left) and in survival percentages (table on the right). $n=3-5$ mice/group. Experiment was done once.

4.4.3 Production of SV40 large T antigen

Next, we also wanted to study how type-2 immune skewing would affect tumour development in a spontaneous prostate tumour model (TRAMP). For this purpose, SV40 large T antigen (SV40 Tag) was chosen as an intracellular antigen for several reasons. Firstly, it is the oncogene used to drive the carcinogenesis in this model; thus, it is implicated in tumour

development. Secondly, SV40 Tag is exclusively expressed in the prostate, offering a minimal risk of side effects in healthy tissues.

Since it was not feasible to purchase a commercial SV40 Tag, we first needed to produce it. To this end, we used the Baculovirus expression system to produce SV40 Tag in Sf9 insect cells. Protein production in insect cells offers a high yield, similar post-translational modification to mammalian expression systems and no risk of endotoxin contamination that is very common when E.coli is used for protein production^{143,144}. Thus, considering the advantages mentioned above, the insect cells were chosen for SV40 Tag production. We infected the Sf9 cells with baculovirus coding for SV40 Tag, and allowed them to express the protein for two days. Since the SV40 Tag is expressed intracellularly, the cells were then lysed and the supernatant obtained after the lysis was used for SV40 Tag purification. Given that SV40 Tag would be used to immunise the mice, we decided not to introduce any tags in the protein that could potentially raise the immune response against them instead of SV40 Tag. Therefore, since we could not use tag-based purification, the SV40 Tag was purified by anionic exchange followed by size-exclusion chromatography (SEC). The elution fractions were tested at multiple stages of purification by western blot for SV40 Tag presence and by SDS-PAGE for general protein purity (data not shown). Unfortunately, a significant amount of protein was lost due to precipitation, and it was detected in the cell pellet after the lysis. However, we successfully purified around 0.8 mg of SV40 Tag, the amount sufficient for our planned *in vivo* work. The western blot and SDS-PAGE analysis of the final sample fractions are shown in **figure 4.5**.

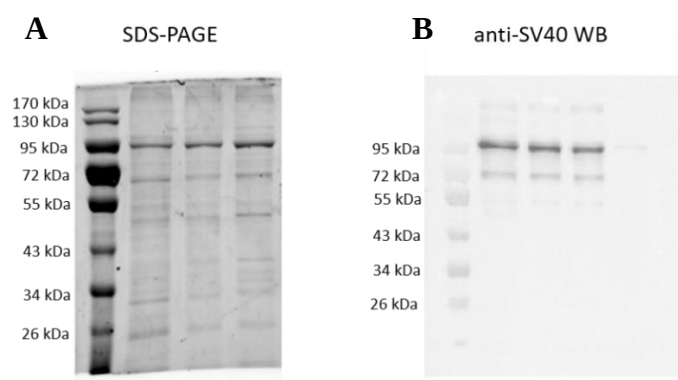


Figure 4.5. SV40 large T antigen was successfully produced in a Baculovirus expression system. SV40 Tag was recombinantly produced in Sf9 insect cells and purified from the cell lysate with anionic exchange and SEC. (A) SDS-PAGE and (B) western blot analysis of the final three sample fractions after purification. The estimated molecular weight of SV40 Tag is around 90 kDa.

4.4.4 Retrovirally transduced T cells express SV40_{IV} TCR

Similarly to the B16-OVA syngeneic model, also in TRAMP mice, we wanted to study the effect of type-2 immune skewing in combination with CD8⁺ T cell adoptive transfer. For this purpose, CD8⁺ T cells had to be engineered to express a TCR that recognises an SV40 antigen. T cells modified with SV40_{IV}-specific TCR have previously been used in the TRAMP model with success¹³⁹. We were kindly gifted with this SV40_{IV}-specific TCR construct by Ton Schumacher's team that initially isolated it. I tested two retroviral packaging cell lines for T cell transduction – Phoenix-Ampho producing amphotropic retroviruses and Platinum-E (Plat-E) producing ecotropic retroviruses. Phoenix-Ampho and Plate-E cells have been transfected with the pMX-SV40 α -IRES-SV40 β coding for SV40_{IV}-specific TCR. Generated retroviruses were then used to transduce the splenocytes pre-activated with concanavalin A and IL-7 for 48h. First, I tested the SV40_{IV} TCR expression by flow cytometry in the packaging cell lines to determine the transfection efficiency of producer cells. This parameter may be a good predictor of the T cell transduction level that will be achieved. My results show that the transfection efficiency reached around 34% for Phoenix-Ampho cells, compared to around 26% for Plat-E (Fig. 4.6 A).

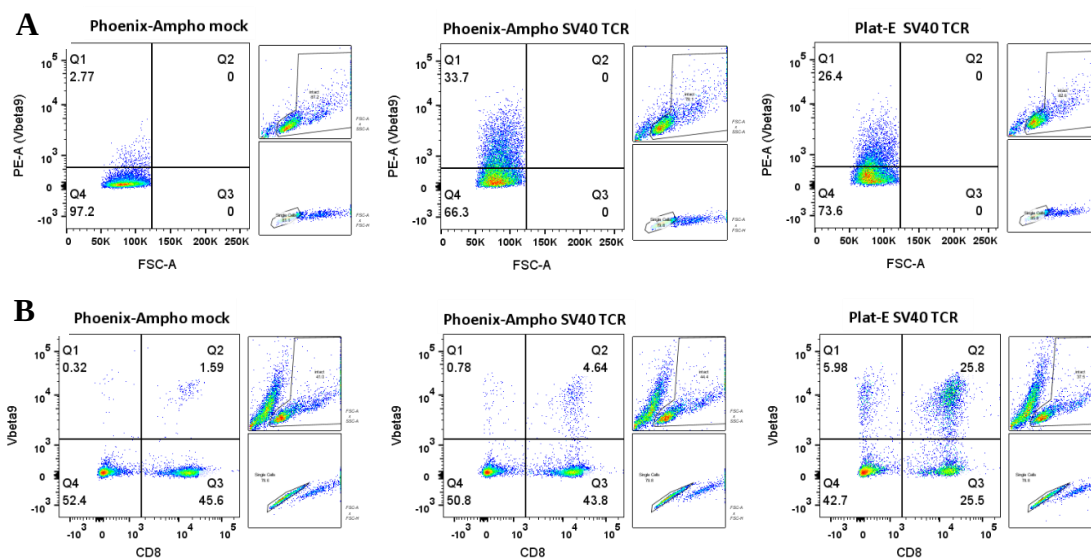


Figure 4.6. The ecotropic retroviral system is superior to the amphotropic one in CD8⁺ T cell transduction. SV40 TCR viral vector produced in either Phoenix-Ampho or Plate-E cells was used to transduce activated splenocytes. FACS was used to assess (A) Transfection efficiency of the producer cell lines. (B) Transduction rate of activated splenocytes (CD8⁺ T cells). V β 9 staining was used to assess the SV40 TCR expression.

Next, I tested the SV40_{IV} TCR expression in the transduced T cells 24h after transduction, as this will be the time point in which they will be used for *in vivo* adoptive cell transfer. The staining included anti-CD8 and anti-TCR V β 9, which is indicative of SV40 TCR expression. The flow cytometry data show that only ~5% of splenocytes were successfully transduced with the retrovirus generated in the Phoenix-Ampho packaging system (**Fig. 4.6 B**). On the other hand, a significantly higher efficiency was achieved with the Plate-E system, reaching around 31% of total splenocytes and about 50% of CD8⁺ T cells (**Fig. 4.6 B**). In conclusion, the ecotropic retroviral system was more successful in transducing the murine T cells, and it will be used in future experiments.

4.4.5 Type-2 immune skewing in combination with CD8⁺ T cell adoptive therapy seem to slow the tumour progression in the TRAMP model of spontaneous prostate carcinoma

Finally, having produced the SV40 Tag protein and tested the T cell transduction protocol, we studied how type-2 immune skewing in combination with CD8⁺ T cell adoptive cell transfer affects tumour development in the TRAMP model. The TRAMP mouse expresses the oncogene SV40 large T antigen under the prostate-specific probasin promoter. Upon reaching puberty, male TRAMP mice develop intra-epithelial neoplasia in the prostate. Adoptive transfer of SV40_{IV} TCR transduced T cells has been reported to delay the tumour development in the TRAMP model when administered at the age of 10 weeks¹³⁹. However, the effectiveness of such treatment is lost when the transfer is delayed to the age of 24 weeks¹⁴⁰. In addition, *in vivo* T cell activation, either by concomitant viral vaccination or by infusing the cells into lymphodepleted mice, was necessary for the adoptive T cell transfer to show effectiveness in tumour control¹³⁹. Therefore, considering the previously published findings, we decided to perform the T cell transfer after week 10, as we did not want to control the tumour growth by ACT alone. In addition, host lymphodepletion was done by total body irradiation (TBI) a day before ACT. The complete treatment scheme of TRAMP mice is shown in **figure 4.7**. First, the mice were immunised with SV40 Tag, using Alum and house dust mite extract (HDM) as adjuvants. This immunisation method induces a type-2 biased immune response with high IgE titers^{145,146}. Ten days after completing the immunisation protocol, TBI was performed, followed by adoptive transfer of SV40_{IV} TCR transduced T cells (around 16 weeks of age). Mice were sacrificed four weeks after ACT, and

the histopathological examination of the prostate gland was done by a trained veterinary pathologist. In addition, blood serum was collected to assess if the immunisation protocol successfully raised anti-SV40 Tag antibodies.

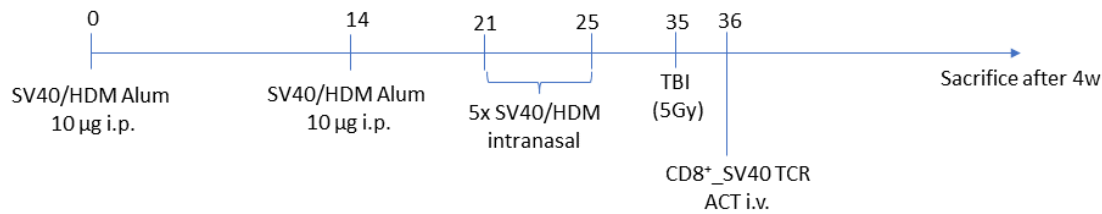


Figure 4.7. *In vivo* experimental scheme for type-2 immune skewing in TRAMP model. The mice were immunised according to the indicated protocol. Ten days later, the host conditioning was achieved with TBI with a 5Gy dose. The following day, mice received the ACT of about 2×10^5 activated CD8⁺ T cells transduced to express SV40_{IV} TCR. The prostate examination was done four weeks after the ACT.

Similarly to the OVA immunisation model, I used ELISA to determine the anti-SV40 IgG and IgE levels, as well total IgE levels. Although high levels of anti-SV40 IgG were detected in the serum of immunised mice, anti-SV40 IgE could not be detected at all (**Fig. 4.8**). Considering that the serum was collected around 6.5 weeks after immunisation protocol was completed, the absence of anti-SV40 IgE in these samples could be explained by the short plasma half-life of IgE antibodies. In addition, total IgE levels were slightly increased in the immunised mice, indicating that IgE response has been induced to some extent (**Fig. 4.8**).

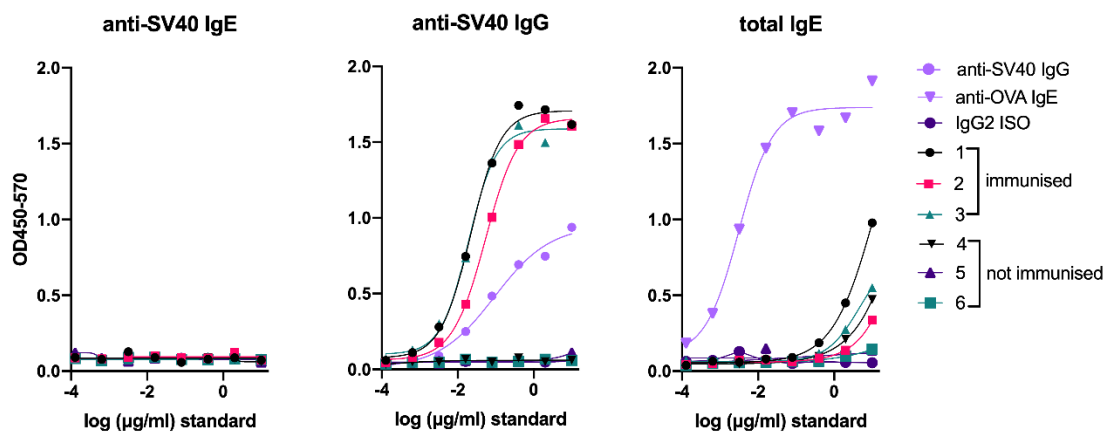


Figure 4.8. Antibody response to SV40 Tag immunisation protocol in mice. The mice were immunised according to the protocol shown in the figure 4.7. The serum was collected at the end of experiment (about 6.5 weeks after the completion of immunisation protocol). ELISA was used to measure the serum levels of (A) anti-SV40 IgE, (B) anti-SV40 IgG and (C) total IgE. n=3 mice/group.

The treatment outcomes were evaluated by histopathological analysis according to the grading system given in **table 4.1** that has been previously described elsewhere¹⁴⁷.

Table 4.1. Histologic grading system for prostate cancer in the TRAMP model

Histologic grade	Features
Normal	Single layer of columnar secretory epithelium with round, basal nuclei, 3-4 cell layers of fibromuscular stroma
Low grade PIN	Variably elongated nuclei with condensed chromatin
High grade PIN	Increasing variability in nuclear shape, very condensed chromatin, appearance of mitotic figures and apoptotic bodies; epithelial stratification and tufting with micropapillary and cribriform structures
Well differentiated adenocarcinoma	Epithelial cells obviously invading through fibromuscular stroma layer and/or the presence of rounded nuclei with condensed chromatin within the fibromuscular stroma which is increased to more than 3-4 cells layers
Moderately differentiated adenocarcinoma	Numerous apoptotic bodies and mitotic figures in compacted, irregularly shaped glands with maintenance of some secretory function
Poorly differentiated adenocarcinoma	Widely variable nuclear shape, clumped chromatin; sheets of cells with little to no glandular formation

The findings are summarised in **table 4.2** and **figure 4.9**, while the representative images are given in **supplementary figure 2**. In the treatment group submitted to immunisation in combination with TBI and ACT, all three mice presented with a well-differentiated carcinoma (stage 3). On the other hand, in the control group that received only TBI and ACT, only one mouse presented with well-differentiated carcinoma, and two mice progressed to moderately differentiated carcinoma (stage 4) and poorly differentiated carcinoma (stage 5). In the untreated group, one mouse had a well-differentiated carcinoma (stage 3), and two mice had moderately differentiated carcinoma (stage 4). It is important to mention that, due to a limited number of TRAMP males available, the untreated group came from a different litter and had to be sacrificed at the age of 19 weeks instead of 20. Taken together, the results seem to indicate that the immunisation in combination with ACT seems to slow the tumour progression in the TRAMP model, with none of the treated animals presenting a stage 4 or stage 5 carcinoma (0/3), compared to the control group in which 2/3 mice did.

However, the number of animals per group (n=3) presents a significant limitation of this study. Therefore, although positive observations have been made, we cannot come to a solid conclusion at this stage.

Table 4.2. The grade of neoplastic changes in the dorsal prostate of TRAMP mice

Findings (stage)	Normal (0)	Low-grade PIN (1)	High-grade PIN (2)	WDC (3)	MDC (4)	PDC (5)
No treatment*	0/3	0/3	0/3	1/3	2/3	0/3
TBI + ACT	0/3	0/3	0/3	1/3	1/3	1/3
Immunisation + TBI + ACT	0/3	0/3	0/3	3/3	0/3	0/3

PIN- Prostatic intraepithelial neoplasia; WDC - well-differentiated carcinoma; MDC - moderately differentiated carcinoma; PDC - poorly differentiated carcinoma

*No treatment group was sacrificed at the age of 19 weeks, whereas the rest were sacrificed at the age of 20 weeks (4 weeks after ACT).

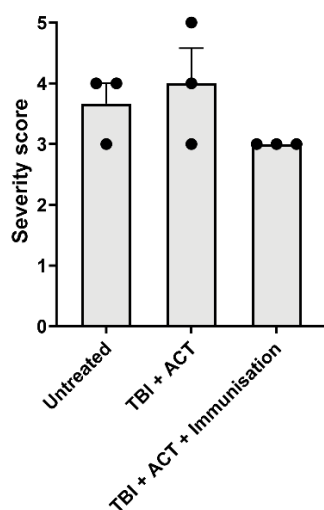


Figure 4.9. Severity score (dorsal prostate cancer stage) of TRAMP mice. Prostates were collected at the end of experiment, fixed in formalin and embedded in paraffin. The hematoxylin & eosine staining was done on cut sections. The histopathological evaluation of prostate tissues was done by a trained pathologist. The treatments groups are indicated (n=3 mice/group).

4.5 Discussion

MCs are a promising novel candidate for tumour immunotherapy as they are present in most solid tumours and easily reachable due to their localisation near blood vessels⁸⁰. However, it would be crucial to enhance their anti-tumour function without promoting the pro-tumorigenic role. Here, we show that this goal might be achieved by targeting an intracellular tumour antigen. The type-2 immune skewing against such antigens led to a significant increase in IgE titers and better tumour growth control when combined with adoptive CD8⁺ T cell transfer. Notably, the beneficial effect was observed in both the syngeneic B16-OVA tumour model and the spontaneous prostate cancer model (TRAMP). Spontaneous tumour models more closely resemble the complex interactions of tumours with their environment in cancer patients¹⁴⁸. Thus, they are extremely valuable in cancer studies.

Since the anti-tumour effect was only achieved with combination treatment comprised of immunisation and ACT, we believe the underlying mechanism must include T_{reg} suppression in the TME. IgE-mediated MC degranulation releases significant amounts of histamine, which has been reported to mediate T_{reg} suppression⁹⁰. Thus, we believe that the raised IgE antibodies induced acute MC degranulation in the TME, leading to T_{reg} suppression and subsequent better tumour control by adoptively transferred CD8⁺ T cells (**Fig. 4.1**). Of note, at this stage, we cannot exclude basophils as potentially involved IgE effector cell population as they also release histamine upon IgE-mediated activation. Our hypothesis regarding T_{reg} suppression in the TME is somewhat supported by our histology results from B16-OVA model, which indicated major lymphocyte infiltration in the treatment group (**Supp. Fig. 1**). However, further studies are needed to confirm such a claim.

Firstly, it would be necessary to repeat the described experiments with bigger animal groups to confirm the reproducibility. Next, since the mice raised high levels of both antigen-specific IgG and IgE antibodies, it would be essential to confirm if IgE antibodies indeed mediated the anti-tumour effect or IgG contributed as well. It is likely that raised IgG antibodies were involved in the anti-tumour effect. Thus, by using the B16-OVA model in IgE-deficient or FcεR-deficient mice, we could confirm if the IgE isotype played a role in the tumour control and if it had synergic effect with IgG. Furthermore, MC involvement could be studied by using the B16-OVA model in MC-deficient mice, in which we would expect to see the loss of treatment

efficacy. Potentially, MC-deficient mice could then be reconstituted with MCs and the treatment efficacy rescued. In addition, by adding H1 histamine inhibitors (e.g. ciproheptadine) to the treatment regimen, the role of histamine could be confirmed. Finally, the T_{reg} involvement should be evaluated. This can be done either by analysing the cellular composition of the tumour material via FACS or histology or by using the mixed bone marrow chimeric approach. The mixed bone marrow chimeric mice could be established by reconstituting the irradiated host with the bone marrow from T_{reg} -deficient mice (80%) and the bone marrow from $Hrh1$ -deficient mice (20%). In such reconstituted mice, all cell types would express the H1 receptor, but T_{regs} , allowing us to confirm if histamine-mediated T_{reg} suppression is the underlying mechanism of enhanced tumour control. Of note, there is a chance that basophils are involved in the anti-tumour control to some extent. This can easily be assessed by studying the B16-OVA model in basophil-deficient mice. Taken together, this set of experiments will reveal if the IgE-MC-histamine- T_{reg} axis is indeed responsible for the observed anti-tumour response.

If the proposed experiments confirm our hypothesis, the results would strongly suggest that IgE-based sensitisation against intracellular tumour antigens may have a realistic therapeutic approach in cancer patients.

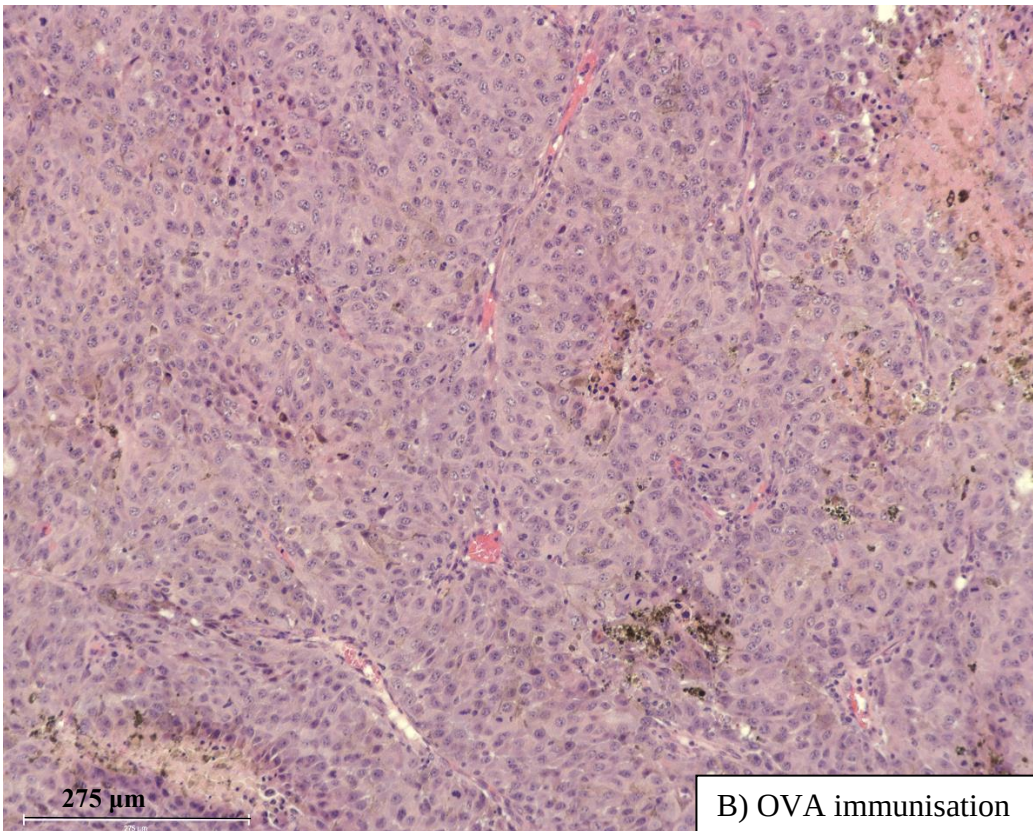
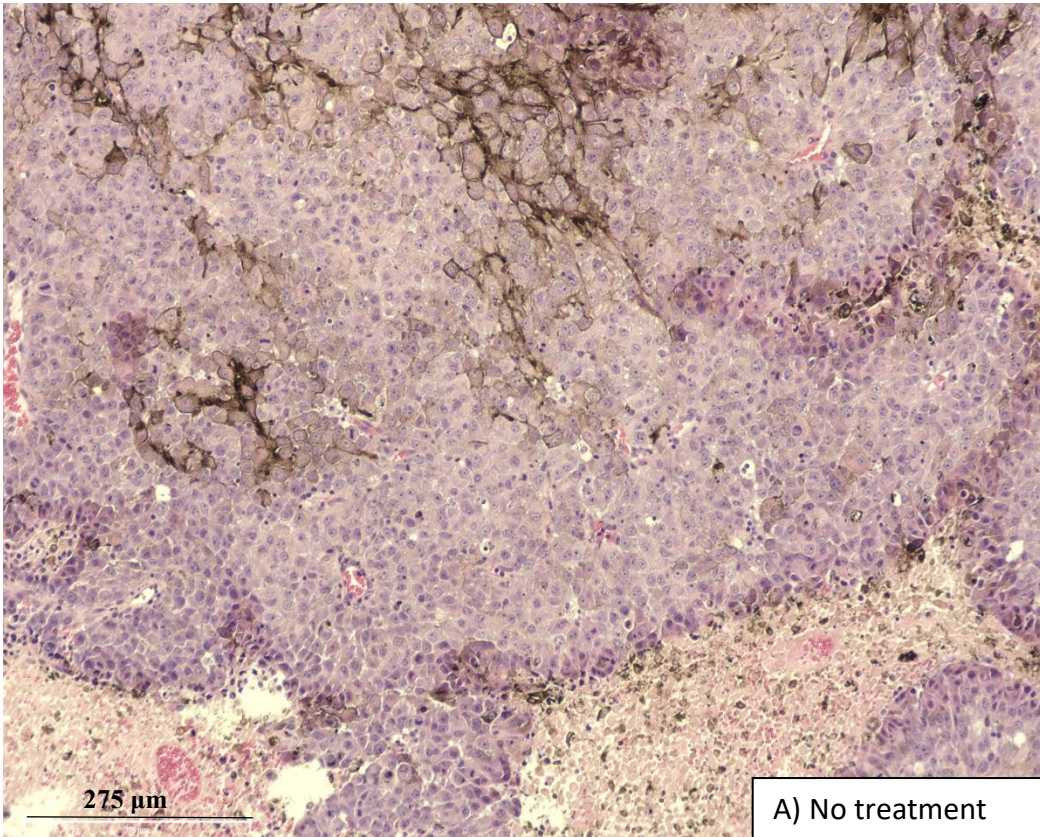
Limitations

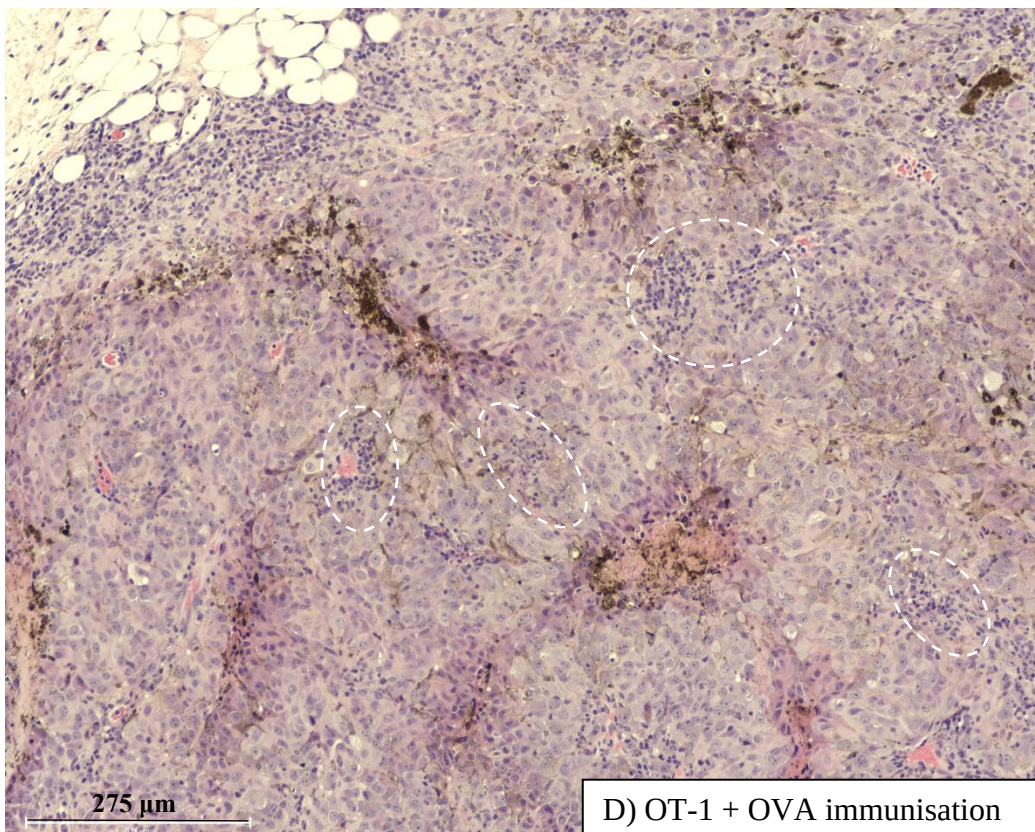
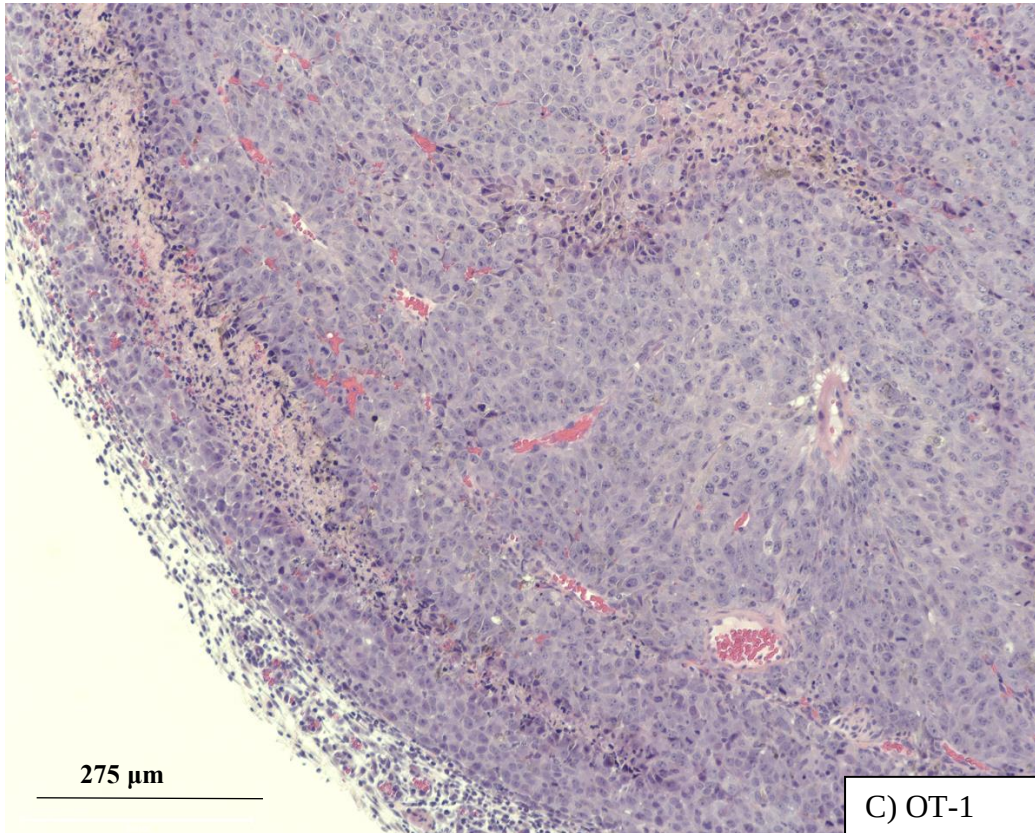
As already mentioned, the major drawback of the presented results is the small size of experimental groups and the fact that the *in vivo* experiments were done only once. However, since a similar anti-tumour effect was observed in both models, syngeneic and spontaneous, we firmly believe that further studies are warranted. Furthermore, I have not looked into different IgG isotypes while evaluating the raised antigen-specific antibodies, primarily due to limited serum volume. However, it would be helpful to know the exact IgG isotype. For instance, IgG1 was reported to be predominantly present in HDM/Alum immunisation protocol, whereas IgG2 levels were not elevated¹⁴⁶. If this is the case in our models as well, we could exclude the IgG-mediated tumour control, as IgG1 is generally considered a non-activating isotype in mice³. Furthermore, our Home Office project licence did not allow us to extract the blood from live animals. Ideally, the presence of antigen-specific antibodies should be evaluated a day or two after the immunisation protocol is finished. In addition, quantifying the IgE in the tumour itself would be important to confirm that IgE effectively reached the

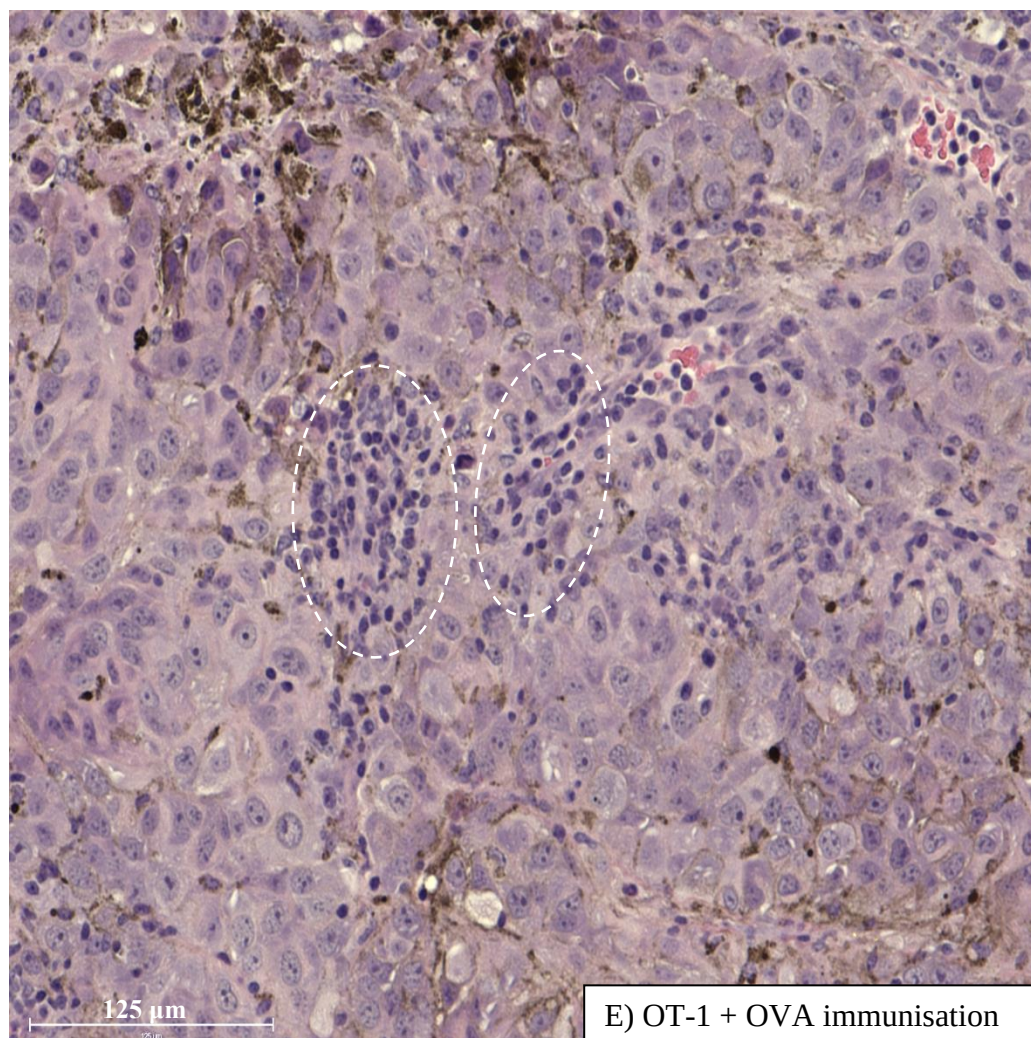
tumour where we hypothesise it induced MC degranulation and subsequent T_{reg} suppression. For example, this could be done by fluorescence microscopy of the tumour tissue with fluorescently labelled anti-IgE antibody. Finally, as already discussed in chapter 3, intratumoral MC degranulation is technically challenging and was not experimentally confirmed. Nevertheless, by using the proposed MC-deficient model and histamine inhibitor, we should be able to confirm the importance of MC degranulation for tumour control.

Conclusions

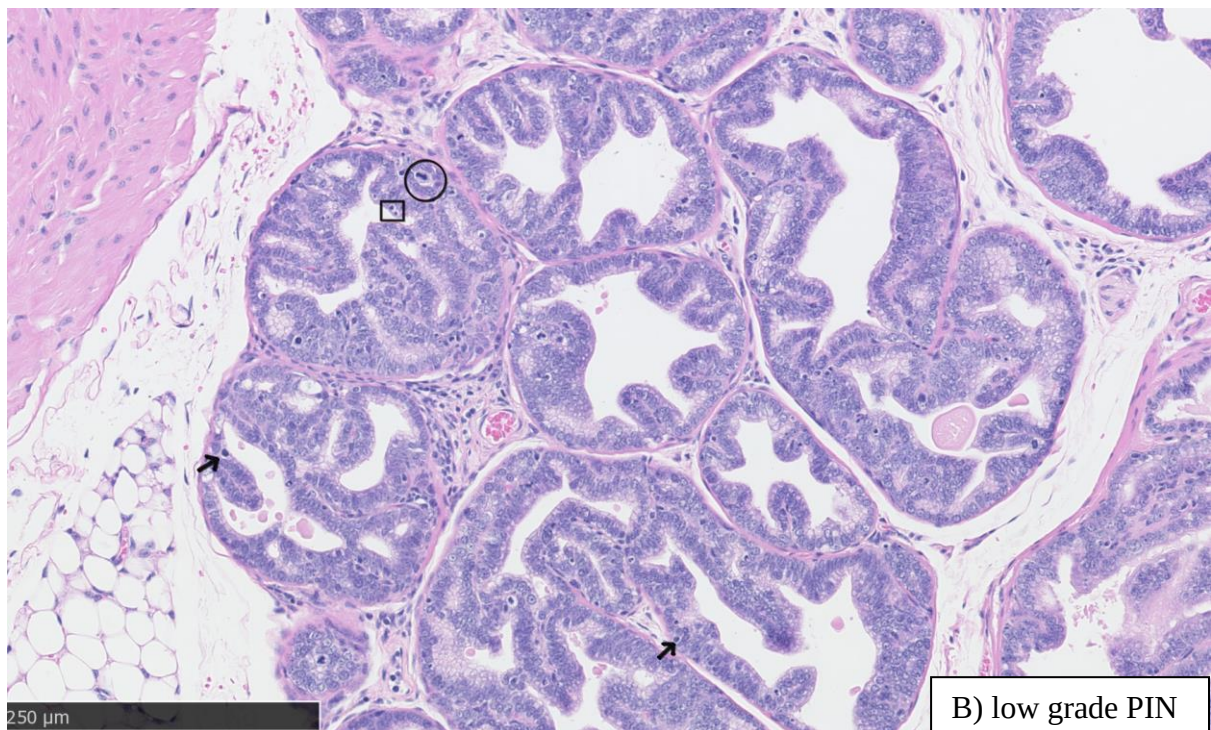
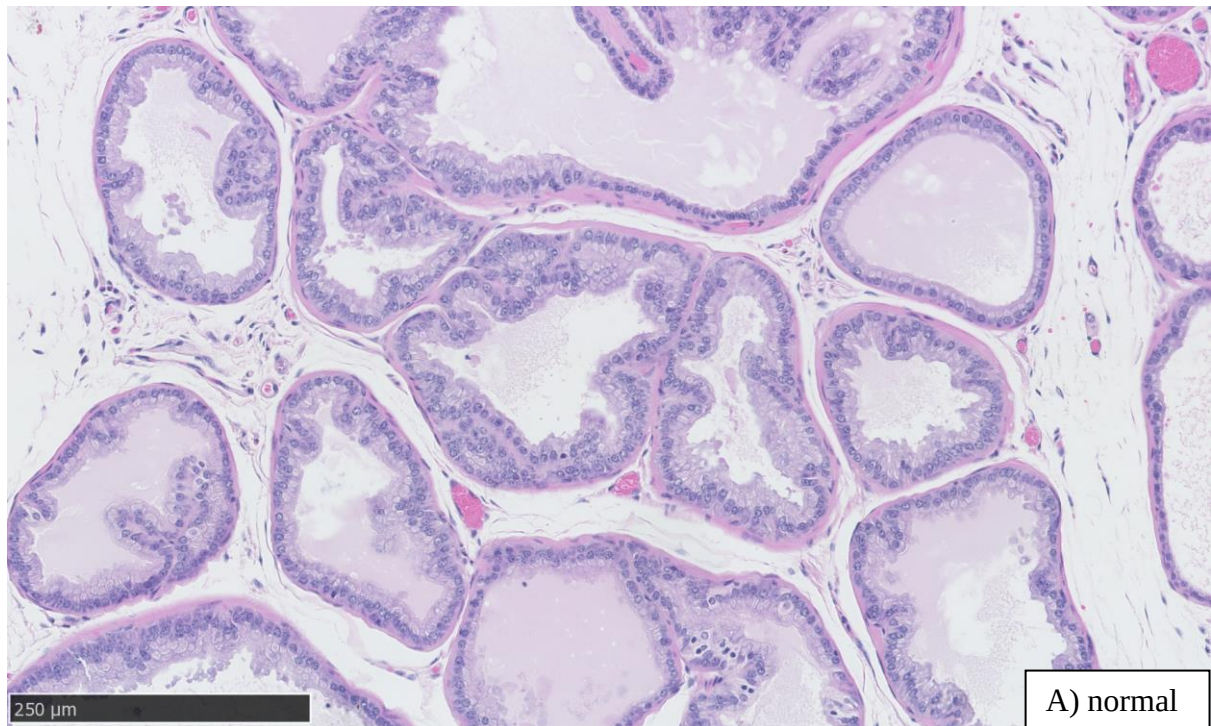
In conclusion, the results presented in this chapter strongly indicate that type 2 immune skewing against an intracellular tumour antigen combined with CD8⁺ T cell adoptive transfer offers superior tumour control in syngeneic and spontaneous tumour models. We suspect that the underlying mechanism may involve IgE-induced MC degranulation, with the released histamine inhibiting T_{reg} suppressor function (**Fig. 4.1**). Multiple experiments were suggested that would be needed to confirm the proposed hypothesis. If confirmed, this novel mechanism could have an important therapeutic application in cancer settings. In addition, the successful activation of anti-tumour MC response would add to our current understanding of IgE tumour control mechanisms, which are until now limited to monocytes/macrophages, dendritic cells and, more recently, NK cells.



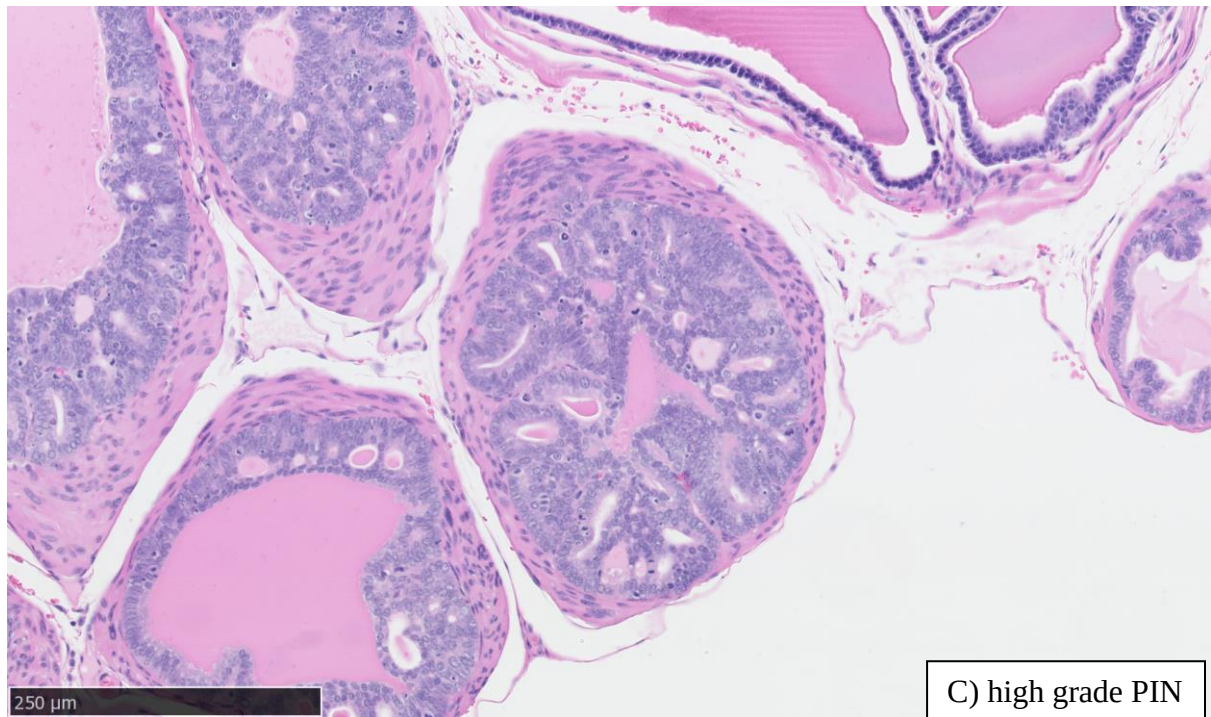




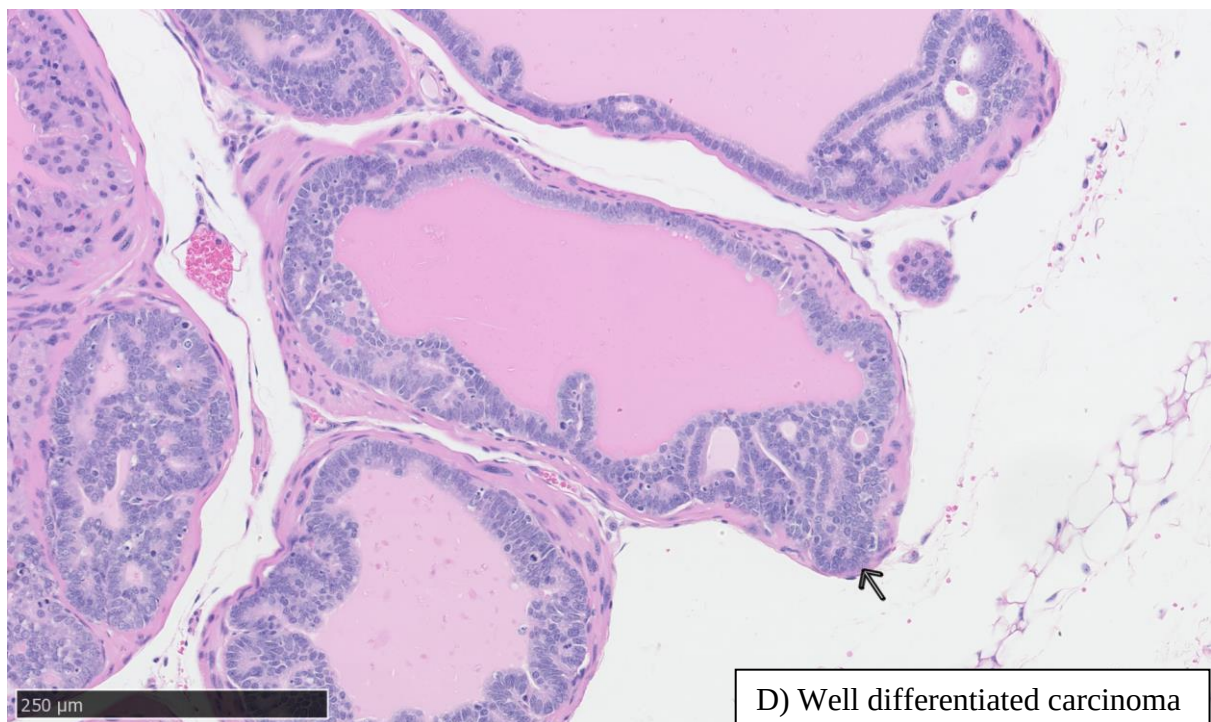
Supplementary figure 1. H&E staining of B16-OVA tumours. The tumours were collected at the end of experiment once the animals reached the endpoint. The formalin-fixed, paraffin-embedded sections were stained with H&E. Representative images are shown. A few areas of marked lymphocyte infiltration have been indicated with white circles on D and E.



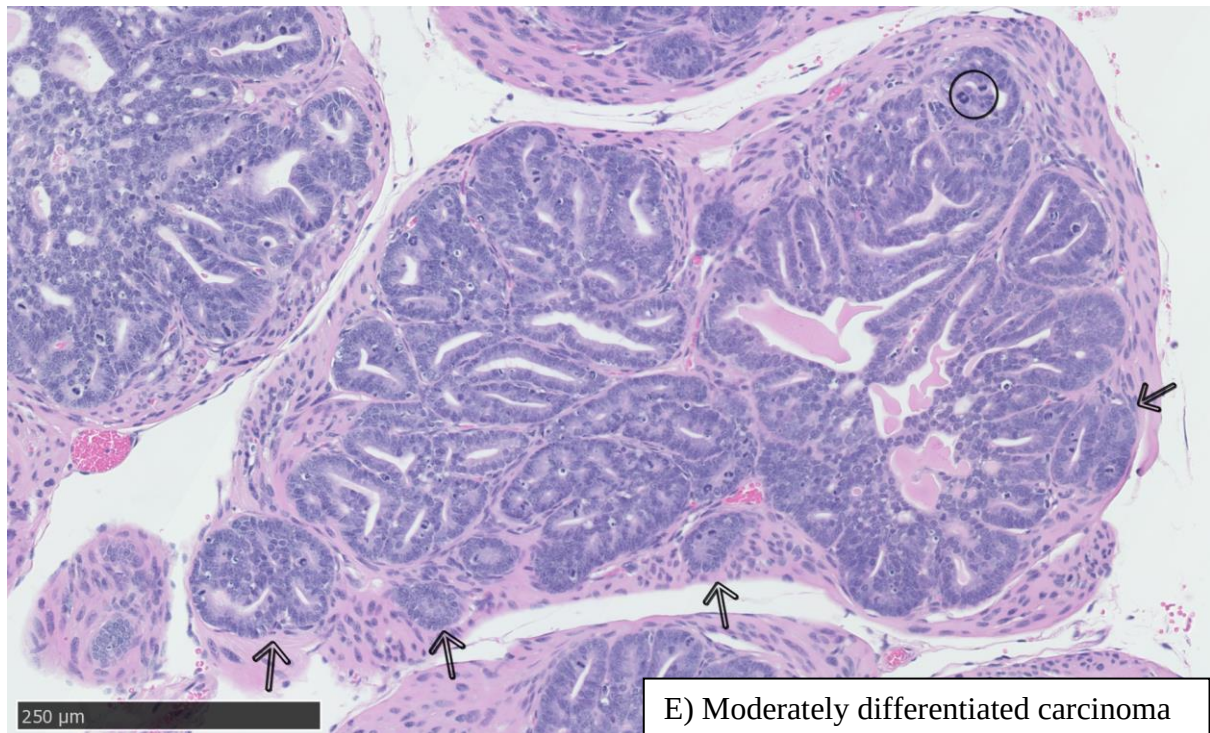
Arrow indicates example of chromatin condensation. Rectangle example of apoptosis (very rare). Circle indicates example of mitosis.



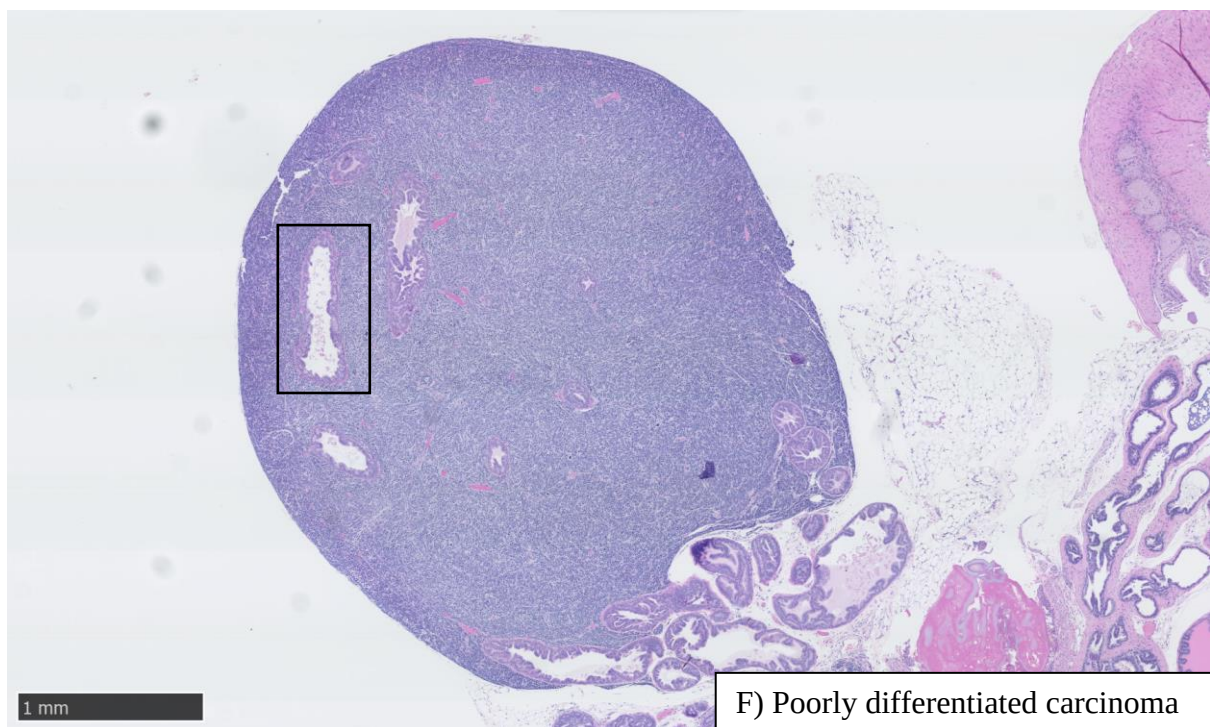
In this instance mitoses and apoptosis are more frequent, and there is formation of cribriform structures without invasion of the fibrovascular stroma.



Similar to high grade PIN, but in this instance, there is also invasion of the fibrovascular stroma (arrow).



Arrows indicate suspected invasion of fibromuscular stroma, circle illustrates mitoses.



Note the effacement of pre-existing structure. There is a trapped pre-existing glandular tissue (rectangle), and the fibrovascular stroma is no longer present at the periphery of the lesion.

Supplementary figure 2. H&E staining of TRAMP dorsal prostates. The prostates were collected at the end of experiment. The formalin-fixed, paraffin-embedded sections were stained with H&E. Representative images of each tumour stage are shown and the histopathological changes described below each image.

5 Concluding remarks

Monoclonal IgE antibodies have shown a great potential in cancer therapy and represent a promising alternative to IgG isotype. So far, macrophages⁵⁰, dendritic cells⁵² and NK cells⁸⁵ have been identified as IgE effector cell types capable of mediating tumour control. This thesis aimed to further explore IgE effector function in cancer settings by putting focus on MCs and basophils - cell populations critical for inducing potent allergic responses, but generally overlooked as potential IgE effector populations in cancer studies. Thus, I used wild type mice in the experiments described in this thesis, as FcεRI expression in wild type mice is limited precisely to these two cell types¹³. Therefore, wild type mice represent an ideal model for studying the possibility of redirecting the allergic reaction against tumour cells.

Since MCs and basophils have been reported to mediate both anti-tumorigenic and pro-tumorigenic roles in cancer^{65,98}, it is crucial to find an optimal way of activating their cytotoxic function without promoting their pro-tumorigenic effects. Based on previously published findings that MC degranulation can mediate tumour control⁹⁴ and T_{reg} suppression⁹⁰, we hypothesised that induction of local degranulation within the tumour with anti-tumour IgE antibodies may be a potent anti-tumour mechanism either through direct cytotoxic effect or reversion of immune-suppressive TME via reported T_{reg} suppression.

In our search for a way of inducing such an allergic reaction within tumours without affecting the detrimental cancer-promoting function of MCs and basophils, we targeted three different tumour antigen types – surface, secreted and intracellular. As shown in **chapter 2**, IgE treatment against a surface tumour antigen did not affect the tumour growth in B16-OVA-Thy1.1 syngeneic model. The lack of any IgE effect could potentially be due to poor contact between tumour cells and poorly infiltrated IgE effector cells. Thus, one could speculate that a surface antigen is not an optimal IgE target for inducing MC and basophil activation. This is not surprising if we take into account that allergens are mostly soluble proteins. Furthermore in this chapter, IgE was also compared to IgG2 and IgG1 of same specificity. My results show that only IgG2a exerted anti-tumour function, reaching the survival rate of about 50%. Although the IgG2a with its high A/I ratio is expected to perform better compared to poorly activating IgG1, their direct comparison in tumour models *in vivo* has been scarce¹²¹. Thus, I provide further evidence confirming the IgG2a superiority over IgG1 *in vivo*. The importance

of this study is further complemented with the finding that none of the tested isotypes (IgE, IgG2a and IgG1) were able to show synergistic effect with adoptively transferred OT-1 cells. Combination therapies consisting of tumour-targeting antibodies and adoptively transferred CTLs have not been previously tested to our knowledge. However, they could potentially have a beneficial effect, if the antibody treatment could attenuate the immune-suppressive state of the TME. Although targeting a surface tumour antigen failed as a potential approach to achieve this goal, in the next chapters I show that IgE is capable of affecting the outcome of T cell ACT in an either harmful or beneficial way depending on the context.

The harmful effect of IgE treatment on the T cell ACT was observed when IgE was directed against a secreted tumour antigen (**chapter 3**). I showed that OT-1 protective effect was completely abolished by anti-OVA IgE treatment in the B16-OVA model in which OVA is secreted from tumour cells. This would fit into the recently published hypothesis by AllergoOncology experts according to which chronic inflammation at primary allergy sites increases the risk of cancer at those sites³¹. One of the examples is the reported increased risk of lung cancer in asthmatic patients³³. Thus, it is tempting to speculate that targeting a secreted tumour antigen, which is constantly released from tumour cells and present in the TME, induced such a chronic activation of MCs and basophils, ultimately leading to worse outcome.

On the other hand, in **chapter 4**, when we targeted an intracellular tumour antigen by immunising the mice under type 2 immune skewing conditions, the T cell ACT exerted superior tumour control compared to ACT alone in both B16-OVA syngeneic model and TRAMP model of spontaneous prostate cancer. Unfortunately, due to technical challenges, we had to resort to immunisation as a way of raising IgE response against an intracellular antigen instead of injecting IgE mAbs. Therefore, it would be crucial to confirm that the observed anti-tumour effect was solely IgE- and not IgG-mediated. If this was the case as we suspect, my findings would have important implications for cancer therapy, as they shed light on potential use of intracellular tumour antigen as IgE targets.

An obvious first choice in that case would be cancer testis antigens (CTAs), which are expressed in a significant number of epithelial solid cancers¹⁴⁹. Their promoters are typically silenced in all cell types except germ cells and placental cells, but become re-activated in tumour cells. Thus, if CTAs are used as treatment targets, minimal side effects are expected

in healthy tissues. A potential candidate could be NY-ESO-1, a cancer testis antigen that has been extensively targeted with cancer immunotherapy¹⁵⁰. In addition to its expression pattern restricted to tumour cells, NY-ESO-1 has been shown to induce spontaneous immune responses¹⁵⁰. Importantly, the data indicate that those natural immune responses against NY-ESO-1 may be suppressed in the TME by T_{regs}¹⁵¹. Thus, it is plausible to hypothesise that anti-NY-ESO-1 IgE antibodies could induce anti-tumour response by reverting that immune-suppressive TME (in addition to exerting direct cytotoxicity via macrophages in human settings). Alternatively, viral antigens derived from oncogenic viruses are also expressed intracellularly in tumour cells and can be used as potential targets¹⁴⁹.

In order for this approach to work, the release of intracellular tumour antigen would need to be induced. However, both chemotherapy and irradiation, which still represent first line therapy for many patients, have been shown to induce tumour cell death and the release of tumour antigens with subsequent induction of anti-tumour immune responses¹⁵². Furthermore, published studies show that the rate of T_{regs} recovery within the first week following these treatments negatively correlates with the efficacy of cancer treatment¹⁵³. We would predict that a combination treatment with IgE-induced tumour-intrinsic MC degranulation should diminish local T_{reg} function in this critical period of time and, thus, enhance efficacy of treatment. Therefore, it is tempting to speculate that combination of chemotherapy or irradiation with IgE mAbs specific for an intracellular tumour antigen could represent a new treatment avenue. Interestingly, Noguchi et al. demonstrated that anti-NY-ESO-1 IgG1 monoclonal antibody treatment combined with chemotherapeutic agents induced potent tumour control characterized by NY-ESO-1-specific CD8⁺ T cell response in mice¹⁵⁴. This study confirms the viability of mAb treatment targeted against intracellular tumour antigens. It would be especially interesting to study this approach in hFcεR1α transgenic mice or rats, where the proposed mechanism of IgE anti-tumour response via MC degranulation could be combined with the activation of already established IgE effector cells such as macrophages, DCs and NK cells.

One potential concern regarding therapeutic use of IgE mAbs is the risk of inducing anaphylactic reaction. However, in our models, we have not observed any signs of anaphylaxis. The IgE treatment has also proven safe in other preclinical models, including mice, rats and primates^{55–57}. Furthermore, the safety was acceptable in humans as well,

where basophil activation test has been identified as an important tool to exclude any patient potentially at risk of suffering anaphylaxis⁵⁴. Thus, we believe that IgE effector cell degranulation can be limited to tumour tissue successfully, but extensive studies would be needed before moving this approach into clinic.

Finally, the successful development of first-in-class human IgE bispecific antibody formats described in the thesis appendix may prove to be an important tool in the future IgE research. Not only could IgE bispecifics be used to improve tumour selectivity but also to improve FcεRI cross-linking. Therefore, we could potentially avoid the need of administering at least two monoclonal IgE antibodies to successfully induce effector cell degranulation. This would in turn significantly reduce the treatment costs.

Altogether, the work presented in this thesis expands the current knowledge of IgE anti-tumour mechanisms, shedding light on the MCs and basophils as potentially important IgE effector cells in cancer. I have shown that the cellular localisation of IgE-targeted antigens critically determines the therapeutic outcome and my work proposes intracellular tumour antigens as viable targets for IgE monoclonal antibody treatment. Thus, my thesis directs the further IgE cancer research toward exploring intracellular antigens further. Animal models with FcεRI expression pattern more similar to humans would be particularly interesting, as they would allow for multiple IgE effector cell populations to be activated and work synergistically, ultimately exploiting the full potential of IgE antibodies as anti-cancer molecules.

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7 Appendix

Published articles:

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Isotype selection for antibody-based cancer therapy

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Summary

The clinical application of monoclonal antibodies (mAbs) has revolutionized the field of cancer therapy, as it has enabled the successful treatment of previously untreatable types of cancer. Different mechanisms play a role in the anti-tumour effect of mAbs. These include blocking of tumour-specific growth factor receptors or of immune modulatory molecules as well as complement and cell-mediated tumour cell lysis. Thus, for many mAbs, Fc-mediated effector functions critically contribute to the efficacy of treatment. As immunoglobulin (Ig) isotypes differ in their ability to bind to Fc receptors on immune cells as well as in their ability to activate complement, they differ in the immune responses they activate. Therefore, the choice of antibody isotype for therapeutic mAbs is dictated by its intended mechanism of action. Considering that clinical efficacy of many mAbs is currently achieved only in subsets of patients, optimal isotype selection and Fc optimization during antibody development may represent an important step towards improved patient outcome. Here, we discuss the current knowledge of the therapeutic effector functions of different isotypes and Fc-engineering strategies to improve mAbs application.

Keywords: cancer, effector functions, Fc tail, isotype, mAbs

Introduction

Monoclonal antibodies (mAbs) have become an increasingly important class of drugs, with a global market comprising a total of 93 mAbs with marketing approval [1], with cancer being their most prevalent target disease [2]. Significant breakthroughs made in the areas of hybridoma technology, phage display and recombinant antibody production have enabled the development of a large variety of specific mAbs of any isotype.

In the case of cancer therapy, most therapeutic mAbs have been designed to interfere with the biological function of their target molecules and Fab arm specificity is, therefore, extremely important for mAb therapeutic efficacy. In addition, the Fc tail, which dictates downstream effector functions of an antibody, also plays an important role. Therefore, the final outcome of the binding of an antibody to its target is also influenced by the chosen isotype (Fig. 1, Table 1). Moreover, Fc- or glyco-engineering of the chosen isotype can be used to

further optimize its effector functions and half-life. In this review we focus on optimal isotype selection for three mAb types receiving much clinical attention, which are according to their mechanism of action: (a) tumour antigen-targeting, (b) immune checkpoint inhibiting and (c) tumour necrosis factor receptor (TNFR) family targeting agonistic mAbs.

Tumour antigen-targeting mAbs

Mechanism of action of tumour antigen-targeting mAbs

The first generation of mAbs approved for clinical application – and still the most common group of mAbs in cancer therapy – consisted of mAbs directly targeting tumour antigens. These tumour antigens are, to a greater or lesser extent, important for the growth, survival and invasiveness of the tumour. The interference with tumour cell signalling pathways affects cell proliferation and leads

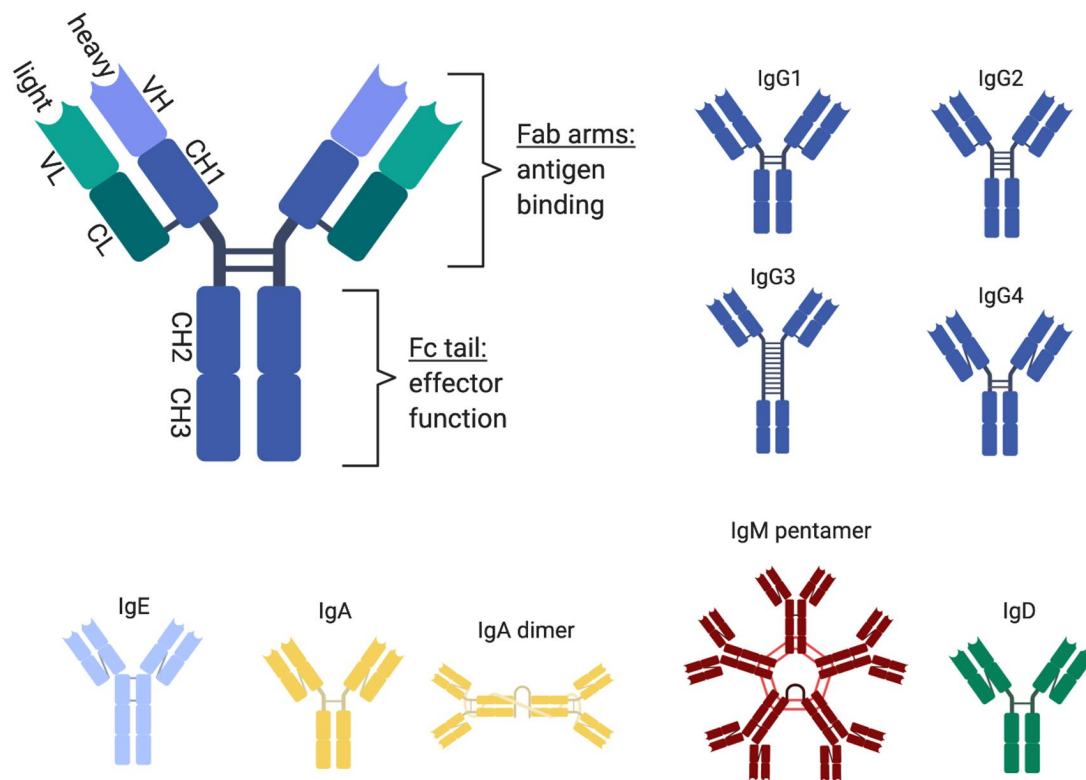


Fig. 1. Antibody structure and isotypes. Human antibodies can be classified into five main isotypes – immunoglobulin (Ig)G, IgA, IgM, IgE and IgD, with IgG and IgA being further divided into the subclasses IgG1, IgG2, IgG3, IgG4, and IgA1 and IgA2, respectively. Overall structural organization of an antibody molecule is similar for all isotypes. It consists of two heavy and two light chains joined by disulphide bonds. Both the heavy and light chains have a highly diverse variable domain (VH and VL, respectively) and one or more constant domains (CH1 CH2, CH3 and CL, respectively). The constant domains of heavy chain are identical for all antibodies of the same isotype/subclass. Antibodies can also be divided into two functional subunits: (1) Fab arm, responsible for the specific binding to the antigen, (2) Fc tail, responsible for the activation of antibody effector functions [complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC), antigen cross-presentation] through interaction with the complement system and binding to Fc receptors present on immune and other cells. A graphical overview of different isotypes and subclasses is shown.

to tumour cell death [e.g. anti-human epidermal growth factor receptor 2 (HER2), anti-epidermal growth factor receptor (EGFR)] [8,9]. However, several observations in humans and mice suggest that Fc-mediated activation of immune cells is an important additional mechanism of action of many of these mAbs [9,10] (Fig. 2). Tumour cell-bound antibodies can bind with their Fc tail to activating FcRs present on effector cells, such as natural killer (NK) cells, macrophages or neutrophils, which then mediate tumour cell lysis [10]. This can occur via release of cytotoxic mediators [antibody-dependent cell-mediated cytotoxicity (ADCC)] or via phagocytosis of tumour cells [antibody-dependent cell-mediated phagocytosis (ADCP)]. In addition, with their Fc tail, antibodies can activate the complement cascade through binding of C1q which can result in tumour cell lysis via several different mechanisms [11]. These include the formation of the membrane attack complex (MAC), that directly induces the lysis of target

cells [complement-dependent cytotoxicity (CDC)], or the attraction of immune cells through the chemoattractive activity of the complement components C3a and C5a. Furthermore, the opsonization by C3b and C4b marks the target cells for complement-dependent cell-mediated cytotoxicity (CDCC) by NK cells, macrophages/monocytes and granulocytes, or for complement-dependent cell-mediated phagocytosis (CDCP) by myeloid cells [11]. Antibody-mediated cell death also leads to the release of tumour antigens and the formation of immune complexes (IC) which facilitate the initiation of anti-tumour T cell responses, sustaining the tumour control and rejection. During this process, binding to FcγRs and activation of complement have been shown to play a critical role in the uptake of IC and cross-presentation of IC-derived tumour antigens by dendritic cells (DCs) *in vivo* [12,13].

In conclusion, in addition to blocking important signalling pathways in tumour cells with their Fab arm,

Table 1. Immunoglobulin (Ig) isotypes mediate their Fc effect via different receptors and activate different immune cells

Antibody isotype	IgG1, IgG3*	IgE		IgA1, IgA2**		IgM
Main effector Fc receptor/ Clq	FcγRIIIa	Clq	FcεRI	FcαRI		Clq
Activated immune cells [3–5]	Macrophages, monocytes, NK cells	Macrophages, monocytes, eosinophils, neutrophils, DCs	Mast cells, granulocytes, macrophages, monocytes, DCs	Eosinophils, neutrophils, macrophages***, monocytes, DCs***		-

*IgG2 and IgG4 are generally considered to have poor Fc effector function, although IgG2 can activate myeloid cells via FcγRIIIa [6]; **IgA2 seems to be more proinflammatory [7]; ***some subpopulations.

tumour-targeting antibodies furthermore deliver their effect through Fc-mediated ADCC, ADCP and CDC. Therefore, an antibody isotype with the highest capacity to induce these effects should show the highest clinical efficacy. We will discuss different strategies to improve immunoglobulin (Ig)G Fc-effector functions, as well as the potential use of alternative isotypes such as IgE and IgA (Fig. 4a).

Optimizing IgG effector function

IgG Fc-effector functions are mediated via complement and FcγRs which are either activating (FcγRI, FcγRIIa/IIc, FcγRIIIa, FcγRIIIb [14]) or inhibitory (FcγRIIb) [15]. As most effector cells co-express both activating and inhibitory FcγRs, the outcome of IgG binding is a result of the relative binding affinity, receptor availability and signalling capacity. The relative affinity of an antibody for its receptors is defined as the activating-to-inhibitory (A/I) ratio [16] (Fig. 3). The concept of the A/I ratio is based on observations in mice which show a high A/I for mIgG2a, a low A/I for mIgG1 and an intermediate A/I for mIgG2b [16]. As a consequence, therapeutic antibodies of the mIgG2a subclass have been shown to clear the tumours more efficiently in many *in-vivo* model systems [17]. Although the differences in the A/I ratio are less pronounced between human IgG subclasses, they also differ in their capability to induce immune responses due to their different FcR binding profile [18,19]. IgG1 and IgG3 bind to all FcRs, but show higher affinity for the activating ones. Thus, they are defined as highly activating with strong Fc-effector function. Conversely, IgG4 binds with similar affinities to most activating FcRs and inhibitory FcγRIIb and is considered as poorly activating. Finally, IgG2 shows overall poor binding to most FcRs, with the exception of the high-affinity H131 FcγRIIIa allele (as discussed later), and has limited Fc-effector function. Therefore, IgG1 and IgG3 are capable of exerting potent effector functions desirable for depleting antibodies, whereas IgG2 and IgG4 are preferred when Fc-mediated cell depletion is to be avoided.

Although less relevant for effector functions, an additional IgG receptor is the neonatal Fc receptor (FcRn) which mediates IgG transport through the placenta as well as IgG cellular recycling, providing IgG with a relatively long serum half-life and thus favourable pharmacokinetic properties [20]. FcRn also binds albumin with similar effects [20] which can be exploited for mAb engineering, as will be discussed later.

Currently, most of the clinically approved tumour-targeting mAbs are of IgG1 isotype, which was shown to be superior to other isotypes and subclasses in inducing ADCC by mononuclear cells as well as CDC *in vitro* [21]. IgG1 achieves most of its Fc-effector functions via FcγRIIIa present on macrophages and NK cells (ADCC, ADCP), as well as via complement activation [22].

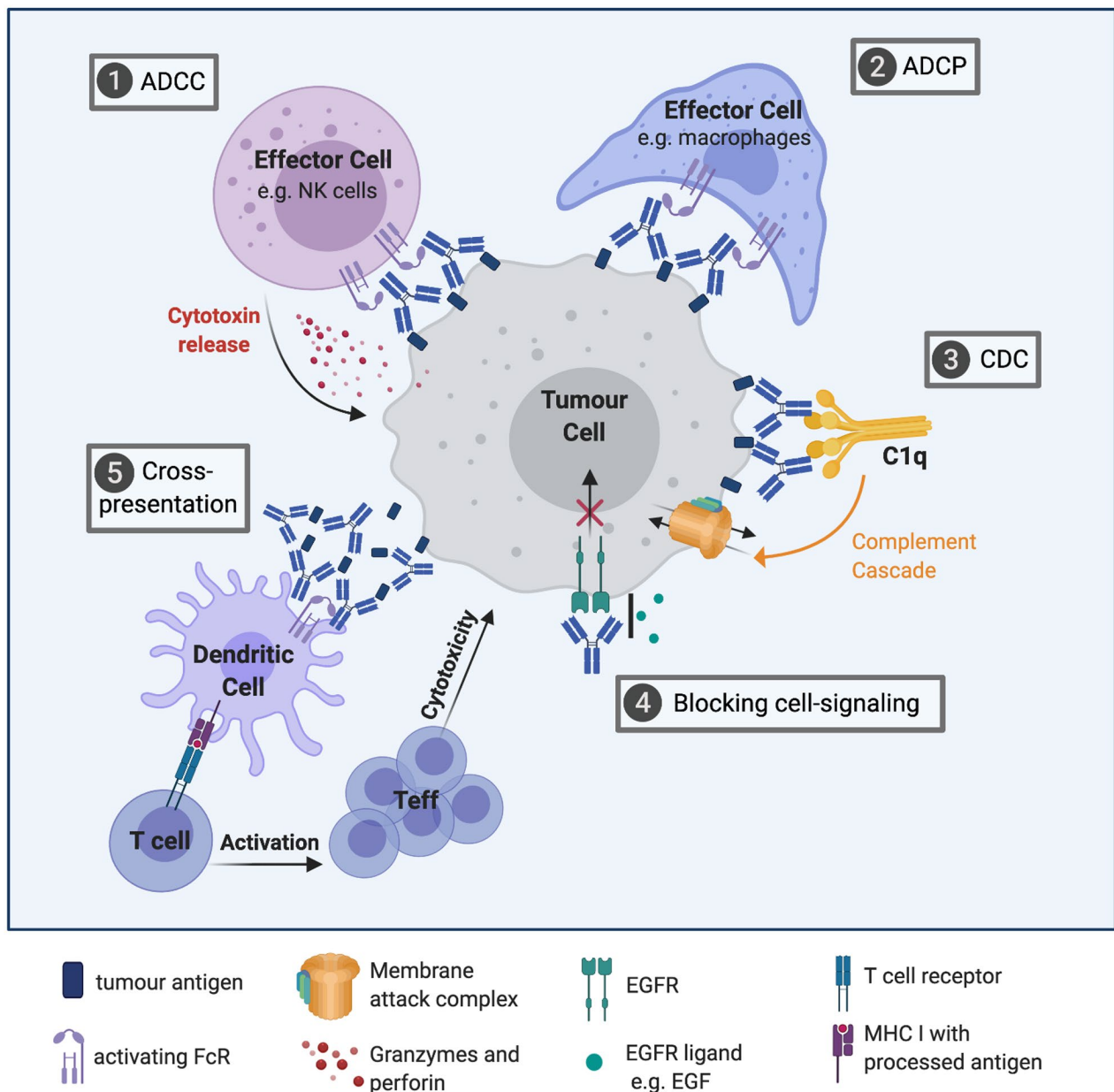


Fig. 2. Mechanisms of action of tumour antigen-targeting antibodies. Tumour antigen-targeting (tumour-depleting) antibodies mediate tumour cell-killing via different mechanisms: (1, 2) activation of immune effector cells [antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP)], (3) initiation of complement cascade [complement-dependent cytotoxicity (CDC)], (4) blocking important signalling pathways in tumour cells and (5) formation of immune complexes inducing enhanced tumour antigen cross-presentation by dendritic cells (DCs), leading to adaptive immune response.

Furthermore, IgG1 shows favourable biopharmaceutical characteristics with regard to production and purification. However, independently of their specificity, all tumour-targeting IgGs used hitherto in the clinic displayed a therapeutic effect only in a subset of patients. Therefore, over the years different strategies have been explored to further optimize tumour-targeting mAbs, many of which focused upon improving Fc-mediated functions.

Improving the A/I ratio. One of the common approaches to improve the IgG Fc-effector functions is to optimize the A/I ratio by increasing affinity for the activating FcγRs, on one hand, and decreasing binding to the inhibitory FcγRIIb on the other hand. One approach to improve the A/I ratio was successfully achieved by glycoengineering. The most relevant modification is afucosylation of N297 glycan, which significantly increased the affinity for FcγRIIIa improving the

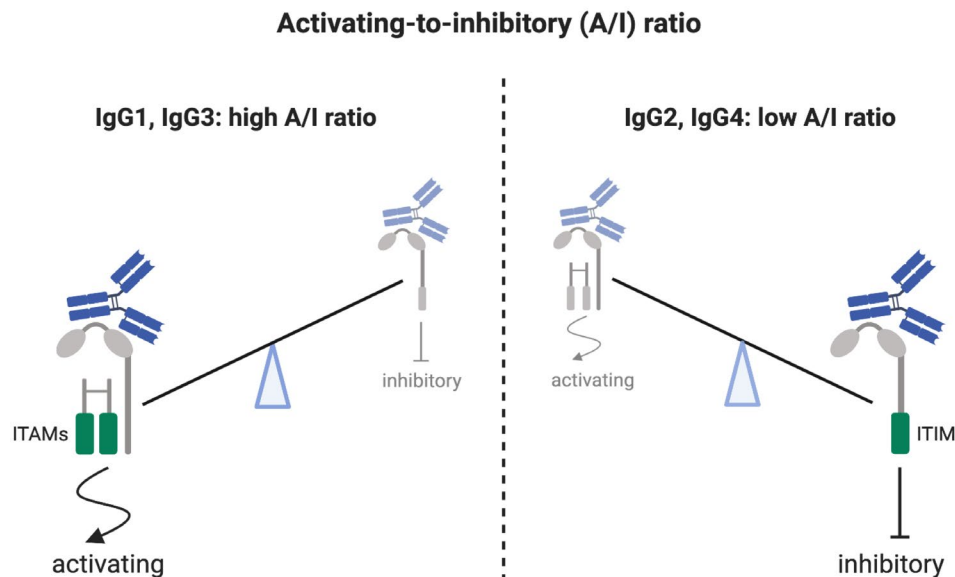


Fig. 3. T. A/I ratio dictates the outcome of Fc-effector function of immunoglobulin (Ig)G antibodies. FcRs can be either inhibitory (FcγRIIb) with immunoreceptor tyrosine-based inhibition motif (ITIM) or activating (FcγRI, FcγRIIa/IIc, FcγRIIIa, FcγRIIIb) [15]. Activating FcγRI and IIIa are associated with the common FcR gamma chain dimer containing two immunoreceptor tyrosine-based activating motifs (ITAMs); FcγRIIa and IIc are not associated with the gamma chain, but contain their own ITAM motif, whereas FcγRIIIb does not contain an ITAM motif and is not always considered as an activating receptor [15]. Expressed mainly on neutrophils, FcγRIIIb has been shown to favour phagocytosis (ADCP) in co-operation with FcγRIIa but, conversely, it has a negative impact on neutrophilic antibody-dependent cell-mediated cytotoxicity (ADCC) by acting as a decoy receptor for IgG, thus competing with FcγRIIa for antibody binding [14]. Cross-linking of an activating receptor with the inhibiting receptor results in down-regulation of the activating signal. Therefore, all activating FcγRs are counterbalanced by one inhibiting receptor FcγRIIb. Differential affinity of IgG for FcRs is defined as activating-to-inhibitory (A/I) ratio.

ADCC effect *in vitro* [23], which was mirrored by improved *in-vivo* anti-tumour responses in mouse models [24]. Two afucosylated mAbs have already received marketing approval [mogamulizumab (anti-CCR4) [25], obinituzumab (anti-CD20)] [26], and several others are currently in clinical trials [27]. Obinituzumab was found to be superior to rituximab (non-glycoengineered anti-CD20) in terms of complete response rate and progression-free survival in various clinical settings [28–30]. Another commonly used strategy to improve the A/I ratio is the introduction of point mutations in the Fc tail [22]. The most promising mAb in this group is margetuximab, an anti-HER2 antibody featuring five point-mutations in its Fc tail resulting in improved binding to FcγRIIIa and FcγRIIa, as well as a decreased FcγRIIb binding [31]. These modifications translated into improved ADCC *in vitro* [31], enhanced anti-tumour activity in mice [31] and higher response rate and progression-free survival in HER2-positive metastatic breast cancer when compared to its non-Fc-engineered analogue trastuzumab [32]. In conclusion, glyco- and Fc- engineered IgG1 mAbs with an optimized A/I ratio appear superior to non-engineered IgG1, due most probably to enhanced ADCC.

Optimizing CDC. CDC has been recognized as an important mechanism of action for some therapeutic mAbs,

such as anti-CD20 [33–35]. Thus, strategies to optimize Fc-mediated complement activation are currently being developed.

Intrinsically, due to its naturally occurring pentameric and hexameric forms, IgM demonstrates the highest capacity for complement activation. However, IgM has received little attention in therapeutic mAbs development, and only a few tumour-targeting IgM mAbs have been evaluated in clinical trials [36], most prominently with PAT-SM6 receiving orphan drug designation by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for multiple myeloma [37,38].

Among IgG subclasses, IgG1 and IgG3 are good complement activators, with IgG3 appearing to be the more potent isotype. Nevertheless, although IgG3 intrinsic problems, such as a short *in-vivo* half-life, have successfully been addressed [39], specific manufacturing issues make it a less attractive candidate for drug development. A way to combine the advantages of both IgG1 (favourable manufacturing characteristics) and IgG3 (enhanced CDC) was achieved through the construction of IgG1/IgG3 chimeric antibodies [40]. The optimal construct, called 113F, combined the CH1 and the hinge of IgG1 with the CH2 of IgG3 and a CH3 which was partly of

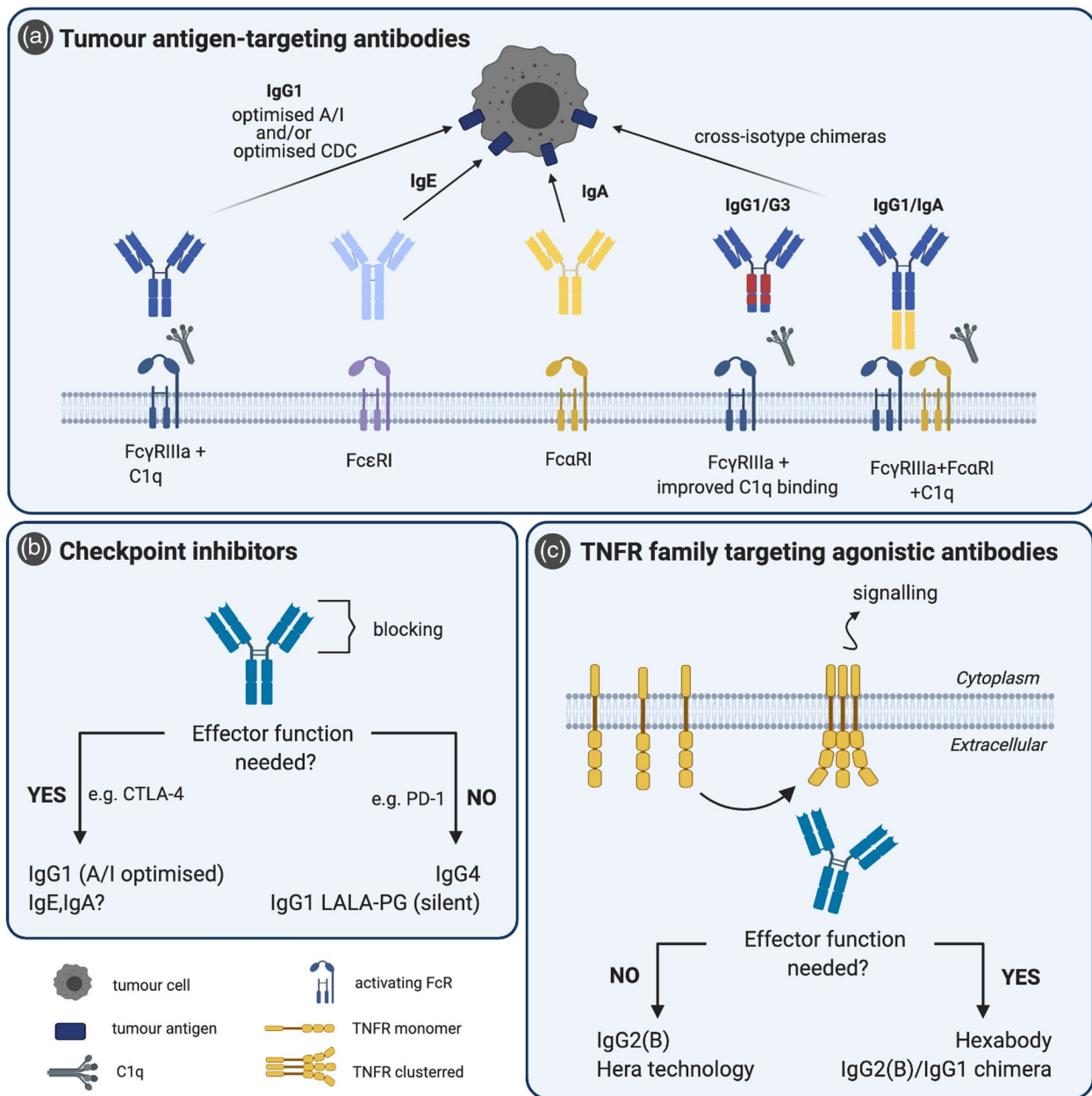


Fig. 4. Optimal immunoglobulin (Ig) isotypes for therapeutic monoclonal antibodies (mAbs). (a) Tumour antigen-targeting antibodies are mainly of IgG1 isotype, which can be further improved by optimizing their A/I ratio and complement activation through Fc- and glyco-engineering. Recently, interest in IgE, IgA and cross-isotype chimeras is rising, as they can offer alternative immune responses against tumour cells and the chimeras combine the advantages of two different isotypes. (b) For checkpoint inhibitors, a functional Fc tail may be either beneficial [anti-cytotoxic T lymphocyte antigen (CTLA-4)] or detrimental [anti-programmed cell death 1 (PD-1)]. If Fc-effector function is needed, isotype selection is similar to depleting antibodies (a). If Fc-mediated effects are unwanted, optimal isotypes are IgG4 (poor Fc effector function) or IgG1 with abrogated FcR and C1q binding, for instance via LALA-PG mutation [86]. (c) Agonistic antibodies target different receptors of the tumour necrosis factor receptor (TNFR) family, which require receptor clustering for initiation of their signalling cascade. Different strategies for achieving receptor clustering are available. In addition, Fc-mediated cell depletion plays an important role in some cases. Prior to selecting the optimal isotype, the need for a functional Fc tail should be considered.

IgG3 and partly of IgG1 origin. The non-fucosylated version of this chimeric antibody showed enhanced CDC and ADCC comparable to non-fucosylated IgG1, in

addition to preserved protein A binding, important for the purification process. The improved efficacy of this chimeric construct was confirmed in cynomolgus

monkeys, where an anti-CD20 113F antibody construct showed greater B cell depletion if compared to IgG1 (both antibodies were non-fucosylated for improved ADCC). This study indicates that the combination of optimized complement activation and A/I ratio represents a promising strategy for the improvement of tumour-depleting antibodies.

Other strategies for enhanced complement activation include the introduction of point mutations to improve IgG1 binding to C1q [22], a key component for the initiation of the complement cascade. Importantly, mutations that potentiate CDC can be combined with ADCP- and ADCC-enhancing mutations in a single IgG1 [41], thus broadening the effector function of these antibodies. Finally, the mutations that favour IgG hexamer formation also significantly enhance C1q fixation and thus CDC [42,43]. However, currently it remains to be seen whether or not these Fc mutations that enhance CDC in *in-vitro* and *ex-vivo* studies translate into improved clinical efficacy.

Use of alternative Ig isotypes

IgE. Several epidemiological studies have suggested a protective effect of some allergies and IgE antibodies against specific types of tumours [44,45], providing a rationale for exploring the potential use of mAbs of the IgE isotype as anti-tumour agents. IgE can mediate its Fc-effector function via two activating receptors: the high-affinity FcεRI and the low-affinity FcεRII. While predominantly expressed by mast cells (MC) and basophils, FcεRI expression can also be found on eosinophils, dendritic cells (DCs) and myeloid cells, although 10–100-fold lower than on fully matured and activated MC [46]. Compared to the IgG class, IgE offers several advantages that can be of interest for cancer therapy. For instance, it shows two orders of magnitude higher affinity for its receptor FcεRI than IgG for its high-affinity receptor FcγRI [47]. Because of such a high FcεRI affinity, IgE is locally retained on the cells expressing FcεRI and has excellent bioavailability in tissues, which is of great importance for treatment of solid tumours. In addition, IgE lacks inhibitory Fc receptors that could cause immunosuppression such as FcγRIIb in the case of IgG [47]. Consequently, the use of IgE antibodies in cancer therapy has been tested both in *in-vitro* and *in-vivo* mouse models, using transgenic hFcεRI mice [48] as well as rats.

Side-to-side studies demonstrated that an IgE mAb targeting tumour-associated antigen was superior to its IgG1 counterpart in terms of survival and reduction of tumour growth [48–50]. Furthermore, it was found that the main anti-tumour effector function of IgE antibodies was mediated by myeloid cells [50,51], and *in-vitro* experimental data showed that monocytes can mediate IgE tumour killing via both ADCC through FcεRI as well as

ADCP through FcεRII [52]. Remarkably, the IgE antibodies both recruited tumour-associated macrophages (TAM) for ADCC and ADCP, but also differentiated them towards activated M1-like phenotype characterized by up-regulation of a tumour necrosis factor (TNF)α/monocyte chemoattractant protein-1 (MCP)-1/interleukin (IL)-10 cytokine signature, suggesting a potential role of IgE in tumour microenvironment (TME) modification [53]. Furthermore, IgE has been shown to facilitate DC cross-presentation of IgE IC-derived antigens, further supporting the anti-tumour effect by inducing a T cell-based anti-tumour response [54–56].

An intrinsic concern regarding IgE therapy is the risk of inducing potentially life-threatening anaphylaxis triggered by degranulation of MC or basophils. Although tumour antigens released into the blood as monomers are not expected to induce cross-linking of FcεR-bound IgE required for degranulation [54], circulating tumour cells expressing multiple copies of targeted antigen would have the potential to induce degranulation. However, no signs of anaphylaxis were found in preclinical models and safety data were satisfactory in both rodents and monkeys [54,57,58], supporting the initiation of the first clinical trial using a tumour-targeting anti-folate receptor alpha IgE mAb MOv18 (NCT02546921). Interim Phase I data from 24 patients support the safety and potential efficacy of MOv18 IgE [59]. Potential to develop systemic allergic toxicity was evaluated by pretreatment skin prick test and *ex-vivo* basophil activation test (BAT). Readily manageable urticaria was the most common side effect. Only one patient experienced anaphylaxis – the only patient with a positive BAT test – indicating that the BAT test might be an important tool to exclude patients with potential risk of anaphylaxis. In addition, although the primary objective was to evaluate the safety and low doses of MOv18 IgE were used, anti-tumour effect was observed in one patient.

In conclusion, the experimental data suggest that IgE might be an attractive Ig isotype to improve the clinical efficacy of tumour-depleting mAbs. Upcoming results of the MOv18 IgE clinical trial will be a major step forward in evaluating IgE-based therapy in human settings.

IgA. Another promising Ig isotype for tumour-depleting mAbs is IgA, which mediates its effector functions through FcαRI. The FcαRI induces activating signals when IgA is encountered as an immune complex; however, it induces inhibitory signals upon monomeric binding [3,60]. FcαRI is highly expressed on polymorphonuclear cells (PMNs), making neutrophils the most relevant cell type for IgA mAb therapy. Neutrophils represent the most abundant cytotoxic cell type in humans. They are armed with a variety of potent cell destruction mechanisms, including the release of

cytotoxic molecules, induction of apoptosis and necrosis. Furthermore, they are well established for their recruitment of other immune cells and phagocytosis [60,61]. Importantly, it has been shown that, compared to cross-linking of Fc γ R, Fc α RI cross-linking is far more efficient in the activation of neutrophils [61–63].

However, *in-vivo* studies are still scarce, due largely to the fact that mice lack a Fc α R homologue. Creation of a transgenic human Fc α R mouse strain [64] allowed *in-vivo* studies in which the anti-tumour effect of IgA antibodies was demonstrated [65,66]. Surprisingly, macrophages were shown to be the crucial effector population for anti-EGFR IgA *in vivo*, leaving the role of neutrophils unclear. Unfortunately, the transgenic hFc α R mouse only partially resolved the lack of a useful model, as human IgA has a very short half-life in mice. Therefore, hIgA mouse pharmacokinetics and exposure were enhanced by attaching an albumin-binding domain to improve FcRn binding and thus the recycling of the antibody [67]. Furthermore, the clearance by the asialoglycoprotein receptor in the liver could be reduced by Fc-engineering [68]. In both cases, increased IgA half-life translated into improved anti-tumour efficacy in mouse models. These strategies may direct more extensive exploration of IgA-based cancer therapies in murine models and might be considered for extending the relatively short serum half-life of IgA mAbs in humans.

Similar to IgG1/IgG3 chimeras, attempts to construct IgG1/IgA chimeras have been made with the intention of combining the advantages of the two different isotypes [69,70]. Binding to FcRn, Fc γ Rs and C1q with the IgG1 part as well as to Fc α R with the IgA part, has been successfully achieved [70]. It provided the IgG1/IgA chimera with an extended half-life, the capability to activate macrophages and complement and initiate recruitment of neutrophils, respectively, resulting in an overall improved cytotoxicity [70]. Thus, the combined effector functions of such a chimeric isotype mAb construct may further improve the clinical efficacy of tumour-targeting mAbs.

Isotypes and patient-tailored medicine

With so many different strategies to improve the downstream effector functions of tumour-targeting antibodies, the question arises as to which approach to follow. It is tempting to speculate that personalized medicine approach may give an answer, by taking into consideration patient-related factors and tumour intrinsic characteristics. For example, two Fc γ R polymorphisms that affect the binding of IgG antibodies have been described: H131R in Fc γ RIIa and V158F in Fc γ RIIIa. The R131 variant shows lower affinity for IgG2 while the F158 variant shows lower affinity for IgG1 and IgG3. The clinical implication of these variants has not yet been

fully resolved, with some studies finding a negative correlation with therapeutic efficacy while others do not [71,72]. Nevertheless, if larger and better-designed studies confirm the negative correlation between lower-affinity FcRs variants and response to IgG antibody treatment, these patients may benefit more from IgE, IgA or mAbs optimized for complement activation, given that these are proven to be effective anti-tumour treatments in the future. With regard to complement optimized mAbs, it may further be important to consider tumour microenvironment (TME) factors, such as pH, that can affect CDC [73] or the expression level of complement regulatory proteins, which allow complement evasion by cancer cells [43]. Furthermore, it has been shown that C-reactive protein (CRP) shares its binding site on Fc γ Rs (I and II) and Fc α RI with IgG and IgA, respectively, whereas it can also bind to C1q [74,75]. Whether CRP can act as a competitive inhibitor for FcRs and complement binding of those antibodies *in vivo* has not yet been studied, but it could have important implications. For instance, patients suffering from chronic inflammatory and neurodegenerative diseases, such as atherosclerosis, type 2 diabetes mellitus or Parkinson's disease, have chronically elevated CRP levels [76] which may interfere with antibody treatment. Thus, for those patients, IgE-based antibody treatment might be an attractive choice. In conclusion, antibody engineering offers a wide range of opportunities to improve effector functions of mAbs, but patient-related factors should also be taken into consideration for optimal isotype selection. This multi-level approach could result in a more effective personalized treatment.

Antibodies targeting immunological checkpoint proteins

A recently identified class of mAbs for cancer therapy are the so-called checkpoint inhibitors. These antibodies do not target the tumour directly but enhance anti-tumour immune responses by targeting immunological checkpoint proteins, such as programmed cell death 1 (PD-1) or cytotoxic T lymphocyte antigen 4 (CTLA-4), or their ligands, such as programmed cell death ligand 1 (PD-L1). These checkpoint proteins are expressed on activated T cells and limit excessive T cell responses. As a means of immune resistance, the ligands of PD-1 are often expressed by tumour cells [77] as well as by myeloid cells infiltrating the tumour microenvironment (TME) [78,79]. Checkpoint blockade leads to enhanced T cell activation [77,80] and, consequently, the clinical introduction of checkpoint inhibitors led to a tremendous improvement of cancer therapy for several different types of cancers.

In theory, checkpoint blocking antibodies do not require Fc-mediated effects, as their main effector function is expected to be derived from blocking the receptor–ligand interaction (Fab-mediated). However, it was found that a functional Fc tail contributed to the therapeutic efficacy of anti-CTLA-4 checkpoint inhibitors in mouse models [81,82]. These studies revealed that whereas both effector T cell (T_{eff}) and regulatory T cell (T_{reg}) populations were increased in lymph nodes after therapy within tumours, specifically the T_{reg} but not the T_{eff} population was decreased. This decrease was only observed with anti-CTLA-4 of the IgG2a isotype (the isotype with the highest A/I ratio in mouse) and appeared mFcγRIV-dependent. The underlying mechanism was found to be caused by a selectively high abundance of macrophages expressing high levels of FcγRIV in tumours but not in lymph nodes [81]. Furthermore, T_{regs} express much higher levels of CTLA-4 than T_{eff} cells and were, therefore, preferentially depleted [83]. These findings point to the importance of the TME for therapeutic mAb efficacy.

There are indications that human anti-CTLA-4 mAbs show the same effect. A recent study confirmed the importance of T_{reg} depletion for human anti-CTLA-4 antibody in a hFcγR mouse model [84]. In addition, in advanced melanoma patients with a high neoepitope burden the authors found a positive correlation between the presence of the high-affinity V158 FcγRIIIa allele and increased response to the CTLA-4 targeting antibody ipilimumab, providing further clinical evidence for the importance of Fc-mediated function. These findings may be relevant to explain why only some patients respond to anti-CTLA-4 therapy and provide further rationale to optimize CTLA-4 mAbs by improving their A/I ratio [85] or switching to IgA or IgE isotypes, given that the microenvironmental requirements for selective tumour T_{reg} depletion are met.

Similarly, it was shown that the binding of anti-PD-L1 mAb to activating FcγRs enhances its therapeutic efficacy in mouse models, due to Fc-mediated depletion of immunosuppressive myeloid cell subsets in the TME [78]. However, although another study confirmed that Fc-mediated depletion of myeloid cells in the TME contributes to the therapeutic effect of anti-PD-L1 antibodies, this effect was found to be dependent upon the mouse genetic background, as it occurred in CT26 tumours transplanted in BALB/c but not MC38 tumours in C57BL/6 mice [79]. The depleted myeloid cell subset was the one with the highest PD-L1 expression, whereas PD-L1 expression on the tumour cells did not contribute to the therapeutic effect of the anti-PD-L1 antibody [79]. Currently, there are three clinically approved anti-PD-L1 mAbs, two of which have a mutated Fc tail with

abrogated FcγR binding (atezolizumab, durvalumab) and one is a wild-type IgG1 (avelumab). As hundreds of clinical trials with these antibodies are currently ongoing, future results might help to resolve the question whether a functional Fc tail improves clinical efficacy of PD-L1 targeting antibodies in humans. If so, a further Fc-effector function optimization might be an appealing step forward.

In contrast to anti-CTLA-4 and anti-PD-L1, a functional Fc tail compromised the activity of anti-PD-1 mAbs *in vivo*. The underlying mechanism of this detrimental effect was the depletion of tumour-infiltrating CD8⁺ T cells, which are characterized by high PD-1 expression [78]. Not surprisingly, two clinically approved anti-PD-1 mAbs are of the IgG4 subclass with poor Fc effector functions. However, as IgG4 still binds to activating FcγRs to some extent, it would be interesting to compare its therapeutic efficacy with that of a mutated mAb with completely abolished FcγR binding [86]. Similarly, antibodies targeting CD47, a 'don't eat me signal' often up-regulated by tumour cells to avoid elimination by myeloid cells as part of CD47/SIRP-α checkpoint pathway, do not require Fc-effector function either [87].

In conclusion, these findings strongly suggest that the cellular composition of the TME as well as the relative expression of the target molecule on different immune cell populations can greatly affect the outcome of checkpoint-blocking mAb therapy. These factors dictate the need for Fc-mediated mechanisms for an optimal therapeutic effect and, thus, the isotype selection for checkpoint inhibitors. (Fig. 4b).

Tumour necrosis factor receptor family targeting agonistic antibodies

Recently, the Fc tail of agonistic mAbs that target specific members of the TNFR family has been shown to play a critical role in their therapeutic efficacy. This class of mAbs is designed to either activate death receptors such as DR4, DR5 and FAS on cancer cells in order to induce cell death, or to activate co-stimulatory receptors such as CD40, 41BB, OX40, GITR and CD27 on immune cells in order to improve anti-tumour immune responses.

TNFRs require trimerization in order to initiate their associated signalling cascade [88]. Therefore, bivalent engagement of these receptors with Fab arms is usually not sufficient for their activation, and additional cross-linking is required. For these antibodies, the interaction with FcγRs functions as an effective scaffold for clustering. Specifically, it has been shown that FcγRIIb represents a dominant scaffold for antibody-mediated TNFR cross-linking and activation of downstream signalling because

of its relatively high expression [89,90]. Consequently, agonistic antibody activity was found to be highly dependent upon successful FcγRIIb engagement in mice [91,92], and Fc-engineered antibodies with improved FcγRIIb binding showed stronger anti-tumour activity [93,94]. However, the expression of FcγRIIb is dynamic and can be down-regulated by particular cytokines [95], rendering the success of FcγRIIb-mediated cross-linking for receptor clustering unpredictable. In addition, effective FcR-engagement by agonistic antibodies was found to be associated with serious hepatotoxicity [96–98], which could potentially be explained by the high expression of FcγRIIb on certain subsets of liver cells [99]. Therefore, new strategies have been explored to improve the agonistic activity of these mAbs independently of FcγR engagement. One of these strategies is the use of hIgG2(B). This compact and highly agonistic conformation of hIgG2 [100] is a consequence of a unique disulphide bonds rearrangement in the hinge region [101]. Compared to hIgG2(A), whose Fab arms are not linked to the hinge via disulphide bonds, hIgG2(B) presents with two disulphide bonds between each Fab arm and hinge, making them more rigid and potentially able to pack TNFR molecules closer together [102]. In line with this finding, the agonistic effect of anti-CD40 hIgG2 antibodies was demonstrated to be FcγR-independent both *in vitro* and *in vivo*. Importantly, it is possible to lock hIgG2 in B conformation via a specific cysteine mutation in CH1 region which allows its recombinant production [100]. Thus, the use of hIgG2(B) is a viable strategy for improving the FcγR-independent agonistic activity of mAbs targeting TNFR family members [103]. Furthermore, isotype switching from hIgG1 to hIgG2 was sufficient to convert an immunosuppressive anti-CD40 antagonistic antibody into a potent agonist with anti-tumour activity [104]. These findings constitute one of the most striking examples of how the choice of the isotype can completely change the activity of a mAb.

Another approach to improve the agonistic activity of TNFR family targeting mAbs, independently of FcγR engagement, is the recently developed HERA platform. HERA is an artificial chimeric molecule which, instead of Fab-arms, has two trimeric TNFR binding domains, fused to an IgG1 Fc backbone with abrogated FcγR binding. The resulting hexavalent molecule is capable of exerting its agonistic activity without FcγR-mediated cross-linking. So far, two HERA molecules targeting CD27 and CD40 have shown promising anti-tumour activity, without significant toxicological signs in pre-clinical mouse models [105,106]. These findings suggest that agonistic HERA molecules may offer improved safety combined with unaltered efficacy and thus an advantageous clinical profile.

The strategies described to improve agonistic activity in a FcγR-independent manner could have an additional advantage, as they prevent unwanted depletion of immune cells expressing the target molecule. However, experiments in mice suggest that the therapeutic effect of some TNFR family targeting agonistic antibodies (such as GITR [107], anti-OX40 [108] or anti-4-1BB) [109] also involved T_{reg} depletion, suggesting that, analogous to anti-CTLA-4, a functional Fc tail might be advantageous. Similarly, some Fc-mediated downstream effector functions may be useful for agonistic mAbs targeting death receptors on cancer cells, as Fc-mediated cytotoxicity and ADCC would act as an additional tumour cell-depleting mechanism and might facilitate cross-presentation inducing an adaptive anti-tumour response.

A few solutions have been proposed to combine the divergent properties, as mentioned above, in a single Ig molecule. For instance, a pentameric IgM antibody with high complement activation capacity has been used to successfully induce DR5 clustering via multi-valent interaction, inducing tumour regression in preclinical models [110]. An alternative approach which takes advantage of Ig multimerization, but avoids IgM manufacturing issues, is the so-called HexaBody technology. It is based on a single point mutation (E430G) in the Fc domain of IgG1 that enhances Fc–Fc interactions upon binding to membrane-bound targets [111]. Consequently, these antibodies have a strong tendency to form hexamers on the target cell, ultimately leading to both high agonistic activity and improved CDC [112]. A combination of different HexaBodies targeting two different epitopes on DR5 is currently in early clinical testing (NCT03576131). Given the enhanced complement activation of HexaBodies, this antibody form could furthermore be attractive whenever tumour cell lysis is intended, such as for classical tumour antigen-targeting antibodies (e.g. anti-CD20), suggestive for the design of an entirely novel type of tumour antigen-targeting antibodies.

In addition to HexaBodies, a highly agonistic anti-4-1BB recombinant Ig with potent Fc-effector function was achieved by combining human IgG2 CH1 and hinge locked in B conformation, with murine IgG2a CH2 and CH3 (the IgG subclass with the highest A/I ratio in the mouse) [109]. In mice, tumour treatment with this chimeric construct induced both T_{eff} stimulation in lymph nodes (strong 4-1BB agonism) and Fc-mediated T_{reg} depletion within tumours, leading to an increased intratumoral T_{eff}/T_{reg} ratio and enhanced survival compared to a wild-type mIgG2a construct [109]. By analogy with the mouse example, a chimera of hIgG2(B) and hIgG1 might be applicable in humans.

In conclusion, important breakthroughs have been made in the design of TNFR agonistic antibodies by making

their activity FcγR-independent. It is precisely the FcγR-independency that may overcome initial problems seen in the clinic, such as severe toxicity and modest efficacy. However, the contribution of Fc-mediated cell depletion to the therapeutic efficacy represents an important consideration for the optimal design of a specific agonistic antibody (Fig. 4c).

Conclusion

The introduction of mAbs into the clinic has fundamentally changed cancer therapy. Nevertheless, it has increasingly become apparent that mAbs mediate their effects via a multitude of different mechanisms of action. Since the selection of the correct Ig isotype was recognized as crucial, much effort has been put into understanding the Fc-mediated effects of different antibody isotypes as well as into Fc-modifications for further improvement of mAbs efficacy. Consequently, several strategies have been developed in order to optimize Fc-mediated effector functions, opening entirely novel opportunities to improve mAbs-based cancer therapy. Furthermore, by considering patient-related factors, such as their immune status, characteristics of the TME or FcγR polymorphism, the isotype selection may either allow for the development of antibodies that are active in a wider range of patients or may allow for the selective use of antibodies tailored towards the individual's needs. Such considerations may lead us one step further to patient-tailored medicine and more effective mAb treatment in the future.

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Disclosures

The authors have no competing interests.

Data Availability Statement

Data availability are not applicable to this review article.

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Technical Note

Purification of murine immunoglobulin E (IgE) by thiophilic interaction chromatography (TIC)

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ABSTRACT

In addition to their known implication in allergy studies, IgE antibodies are becoming an increasingly interesting antibody class in cancer research. However, large-scale purification of IgE antibodies still poses substantial challenges, as they cannot be purified using techniques commonly used for other immunoglobulins such as protein A or protein G chromatography. Here, we have developed and optimised a gentle and simple IgE purification method based on thiophilic interaction chromatography (TIC). IgE binds to the thiophilic resin in presence of 1.2 M ammonium sulfate and is eluted in low salt concentration. Monomericity of purified antibodies ranged between 54 and 73%. Preparative size-exclusion chromatography was thereafter performed to further improve the purity, which reached >95% in the final product. The overall recovery was around 30%. The purification method was tested on both hybridoma-produced and recombinantly produced IgE antibodies with reproducible results. In addition, the antigen binding activity of purified IgE antibodies was preserved, as shown by binding ELISA. Purification by TIC is cheap, gentle in terms of pH to preserve IgE folding and function, and universal as any IgE antibody can be purified irrespective of the species of origin or affinity. Potentially, it could be used for purification of other antibody isotypes as well, when gentle conditions are required.

1. Introduction

Antibodies are widely used in research, diagnostics and therapeutic settings and while the IgG class is the most common isotype, the interest in other antibody classes is rising. Among them, IgE antibodies have been found to play an important role in allergy and parasitic infections, but are also being investigated as potential anti-cancer therapy (Jensen-Jarolim et al., 2017). The first clinical trial of an IgE antibody in oncology settings is ongoing (NCT02546921) due to its superior efficacy in ovarian cancer models compared to its IgG₁ counterpart (Karagiannis et al., 2003). Therefore, given the increasing interest in IgE antibodies, there is also a need for a robust and reliable IgE purification method.

Purification is an important step during antibody production. Ideally, it is cost-effective, yields a pure product with high recovery and preserves biological activity of the antibody. Most commonly used

methodologies such as protein G or protein A chromatography cannot be used for IgE purification, because neither the protein G nor protein A bind to IgE antibodies (Peng et al., 1994). Several other techniques have been described for IgE purification, such as ammonium sulfate precipitation (Reali et al., 2001), hydrophobic charge induction with MEP (Mercapto-Ethyl-Pyridine) HyperCel sorbent (Dodev et al., 2015) and affinity chromatography with either anti-human IgE antibody (Gould et al., 1999), antigen or HiTrap KappaSelect resin (Vigor et al., 2016). However, all of them have certain disadvantages as they may require elution under low pH conditions (MEP HyperCel, KappaSelect) or can only be used for specific IgE antibodies (affinity-based methods). For example, KappaSelect used for the purification of the IgE which is currently in clinical trial (Vigor et al., 2016) can only capture antibodies with kappa light chain.

Thiophilic interaction chromatography (TIC) was first described in 1985 (Porath et al., 1985), and has been successfully used for IgG, IgM,

Abbreviations: CE-SDS, Capillary electrophoresis sodium dodecyl sulfate; Ig, immunoglobulin; OVA, ovalbumin; Prep SEC, Preparative size-exclusion chromatography; UPLC-SEC, Ultra-high performance liquid chromatography-size exclusion chromatography.

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and IgY purification (Boschetti, 2001). It is based on the principle that immunoglobulins interact with the ligand that contains a sulfone group proximal to a thioether group in the presence of high lyotropic salt concentration. After this salt-promoted adsorption, the immunoglobulins are eluted in low salt concentration (Porath et al., 1985; Boschetti, 2001). This method offers certain advantages, such as elution under neutral pH conditions which reduces aggregate formation and preserves biological activity of the antibody (Boschetti, 2001) and, in addition, the possibility to purify any IgE antibody irrespective of species or antigen affinity. Due to the impossibility to use protein A or protein G based methods and considering the above-mentioned advantages of TIC, we decided to develop and optimise a TIC method for IgE purification.

Optimisation included various factors including the salt concentration that affects the strength of resin-ligand interaction, as well as the column size and sample loading flow rate. The last two determine the residence time of the sample in the column, i.e. the duration of interaction between the antibody and the resin. The optimised purification method was then employed to purify murine IgE from hybridoma supernatant. Finally, the method was employed to purify murine IgE antibodies that were produced in a recombinant production system. To our knowledge this is the first report on thiophilic interaction chromatography for IgE purification.

2. Materials and methods

2.1. Cell culture

Hybridoma cells, 2C6, were kindly provided by Prof. Lester Kobzik (Harvard) and were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Biowest), 1% penicillin/streptomycin (Gibco), 50 μ M 2-mercaptoethanol (Gibco), 1% HT supplement (Gibco) and 1% serum-holding T24-CM (in house supernatant from T24 cells containing IL-6). FreeStyle™ 293 cells (Invitrogen) were grown in FreeStyle™ 293 Expression Medium (Invitrogen). CHO-K1 cells (ATCC Cat# CCL-61) were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco), supplemented with 10% Fetal Calf Serum (Biowest) and 1% penicillin/streptomycin (Gibco). ExpiCHO-S™ cells (Gibco) were grown in ExpiCHO™ Expression Medium (Gibco). Culture conditions were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Antibody production by hybridoma

Anti-OVA IgE antibodies were obtained from 2C6 hybridoma. In brief, the cells were seeded to shaking erlenmeyer flask with the density of 5×10^5 cells/mL and cultured for 7 days in hybridoma serum free culture medium (H-SFM, Gibco) with 1% penicillin/streptomycin and 1% serum-free T24-CM at 37 °C, 5% CO₂ and 80 rpm. On day 7 post-transfection, the cell suspension was collected and centrifuged for 10 min at 2700 g. The supernatants were filtered over a 0.22 μ m filter and stored at 4 °C.

2.3. Recombinant antibody production

The OX7 hybridoma (ECACC Acc.No 84112008), which secretes anti-Thy1.1 IgG antibody, was sequenced to determine the heavy and light chain variable domains, respectively (LakePharma). Next, those sequences were used to engineer chimeric mIgE and mIgG1 heavy and light chains. The DNA constructs encoding for anti-Thy1.1 heavy and light chains were *de novo* synthesized (GeneArt). Anti-Thy1.1 IgE and IgG1 were recombinantly produced in FreeStyle293 cells and ExpiCHO-S™ cells, respectively. Briefly, cells were transfected with pcDNA3.1.(+) expression vectors encoding corresponding heavy and light chains (1:1 ratio), using 293fectin reagent (Invitrogen) or ExpiFectamine (Gibco) according to the manufacturer's recommendation, respectively. The cells were incubated for 7 days at 37 °C, 8% CO₂ at 120 rpm or 90 rpm,

respectively. On day 7 post-transfection, the cell suspension was collected and centrifuged for 15 min at 2500 g. The supernatants were filtered over a 0.22 μ m filter and stored at 4 °C.

2.4. Purification of anti-Thy1.1 IgG1

The Expi-CHO-S™ supernatant was mixed with a pre-determined amount of MabSelect SuRe LX resin (GE Lifesciences) and rotated overnight at 4 °C. Following overnight capturing, the bound antibody was purified from the resin by affinity chromatography using Pierce™ Centrifuge Columns (ThermoFisher Scientific) and re-buffered to PBS using PD-10 Desalting Columns (GE healthcare) according to the manufacturer's instructions.

2.5. Sandwich ELISA for IgE quantification

A 96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 0.15 μ g/mL of goat anti-mouse IgE in PBS (Southern Biotech, 100 μ L/well) overnight at 4 °C. Blocking was done with 1% BSA in PBS for 1 h at 37 °C. Next, supernatants containing IgE antibodies were added in serial dilutions. Initially, before serial dilutions, 2C6 hybridoma supernatant was diluted 4 $\times$, whereas FreeStyle293 supernatant containing anti-Thy1.1. IgE was initially diluted 2 $\times$ with cell culture medium. Mouse IgG1 kappa was used as negative control (clone P3.6.2.8.1). Mouse IgE (Bio-Rad cat# PMP68) was used for the standard curve. The incubation was performed at room temperature for 1 h. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). The reaction was stopped after 15 min with 0.5 M H₂SO₄ and absorbances were read at 450 nm and 620 nm. All samples were tested in duplicate.

2.6. Thiophilic interaction chromatography

The required amount of salt was added gradually to the supernatant containing IgE antibodies to avoid precipitation. The samples were put on a tube roller until the salt was completely dissolved. Samples were then filtered through a 0.22 μ m filter to prevent clogging of the resin. Column purification was performed using an ÄKTA pure instrument (GE LifeSciences). The thiophilic resin packed column (G Biosciences cat# 786-268, 6% highly cross-linked agarose as support) was equilibrated with equilibration buffer (20 mM sodium phosphate, 1.2 M sodium sulfate, pH 7.5), after which the previously prepared sample was loaded. Residence time was calculated from the formula: Residence time (min) = [bed height (cm)/flow velocity (cm/h) during sample loading] $\times$ 60. The column was then washed with equilibration buffer until the absorbance reached baseline. The isocratic elution was performed using 20 mM sodium phosphate, pH 7.5. The collection was done starting at >50 mAU and ending with <50 mAU. The column was regenerated with 20 mM sodium phosphate, 30% propan-1-ol, pH 7.5 and stored in 20% ethanol. Antibody concentration was quantified using NanoDrop spectrophotometer at 280 nm (extinction coefficient 1.6) and the samples were stored at 4 °C.

2.7. Ultra-performance size-exclusion liquid chromatography (UPLC-SEC)

If necessary, the samples were concentrated prior to the analysis with Microsep Advance 10 K centrifugal device (Pall Corporation). Centrifugation was performed at 3000 g for 10–20 min. Monomericity of purified antibodies was determined using an ACQUITY UPLC Protein BEH SEC column (Waters, Cat# 186005225) in an Acquity H-class Bio system. Separations were carried out in 50 mM Sodium Phosphate, 0.2 M Sodium Chloride pH 7.

2.8. Preparative size-exclusion chromatography (prep SEC)

The samples were concentrated by using a Microsep Advance 10 K centrifugal device (Pall Corporation) to a final volume of 6–7 mL. Prep-SEC based fractioning was done using a HiLoad 26/600 Superdex 200 prep grade column (GE28-9893-36, $V = 319$ mL, $2.6\text{ cm} \times 60\text{ cm}$) using an ÄKTA pure instrument. Equilibration was done overnight with PBS for 2 column volumes, after which the sample was loaded onto the column with a velocity of 25 cm/h (2.212 mL/min). The antibody was eluted with PBS and collected in 2 mL fractions. Concentrations were quantified by spectrophotometry using NanoDrop and fractions were analysed on UPLC-SEC as described above.

2.9. SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

An amount of 2 μg of each antibody was diluted in Laemmli Sample Buffer (Bio-Rad) for analysis under non-reducing conditions or Laemmli Sample Buffer containing 10% β -mercaptoethanol for analysis under reducing conditions. Samples were heated either at 95 °C for 5 min (reduced) or at 90 °C for 2 min (non-reduced). Samples and EZ-Run™ Prestained Rec Protein Ladder (Fisher BioReagents) were subjected to either 10% polyacrylamide gel at 120 V (reduced samples) or 8% polyacrylamide gel at 100 V (non-reduced samples). Gels were stained using GelCode™ Blue Safe Protein Stain (Thermo Scientific), fixed for 30 min in buffer containing 40% ethanol and 10% acetic acid, destained with distilled water and scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

2.10. Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)

The purity of IgE antibodies was tested by capillary electrophoresis sodium dodecyl sulfate (CE-SDS). CE-SDS analysis was carried out on a CE system PA800 Plus machine (Beckman Coulter). Samples were diluted to 1 mg/mL with 10 kDa internal standard and iodoacetamide in SDS-MW sample buffer and heated to 70 °C for 10 min. 95 μL were transferred into sample vials and loaded into the machine. Separations were performed in a bare-fused silica 50 μm I.D capillary at 22 °C. Effective separation length was 20 cm, run time 40 min and antibody fragments detected at a wavelength of 220 nm. The capillary was flushed with 0.1 M HCl, then 1 M NaOH, then water and run buffer before sample loading at 5 kV for 20 s. Noise was recorded for 3 min from the run buffer. Data analysis was carried out using 32Karat software (version 9.2).

2.11. OVA binding ELISA

A 96-well MaxiSorp plate (ThermoFisher Scientific) was coated with 5 $\mu\text{g/mL}$ of rabbit polyclonal anti-OVA in PBS (EMD Millipore, 100 μL /well) overnight at 4 °C. Blocking was done with 1% BSA in PBS for 1 h at 37 °C. Next, PBS (blank) or 0.08–10 $\mu\text{g/mL}$ of chicken ovalbumin (Sigma A5503) were added. The incubation was performed at room temperature for 1 h. Next, purified anti-OVA IgE was added and the plate was incubated for 1 h at room temperature. Finally, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000 in 1:1 1% BSA PBS/PBST) was added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). The reaction was stopped after 15 min with 0.5 M H_2SO_4 and absorbances were read at 450 nm and 620 nm. The samples were tested in duplicate.

2.12. Thy1.1 transient transfection and cell ELISA

An amount of 24 μg of pcDNA3.1(+)-Thy1.1 plasmid was transfected into CHO-K1 cells (100 mm Petri dish, 80% confluent) using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. The following day, cells were plated into a 96-well plate (50,000 cells/well). Two days after transfection binding

ELISA was performed. The cell supernatant was discarded and either anti-Thy1.1 IgE or IgG1 were added in serial dilutions. The incubation was performed at room temperature for 1 h. Next, goat anti-mouse IgE-HRP conjugate (Southern Biotech, 1:4000) or goat anti-mouse IgG Fc-HRP (Jackson Immuno Research 1:5000) in 1:1 1% BSA PBS/PBST were added for 45 min at room temperature. Immunoreactivity was visualized with TMB Stabilized Chromogen (Invitrogen). Reactions were stopped after 15 min with 0.5 M H_2SO_4 and absorbances were read at 450 nm and 620 nm. All samples were tested in duplicate.

3. Results

3.1. Optimisation of salt concentration and residence time for murine IgE purification by TIC

In order to optimise factors affecting the binding of IgE antibodies to thiophilic resin, 2C6 hybridoma supernatant at a concentration of about 128 mg/L of anti-OVA mIgE antibody was used as starting material (Fig. S1A). The Ig binding capacity of the resin as indicated by the manufacturer was not exceeded in any of the conditions. Antibody recovery rate was improved when the ammonium sulfate concentration was increased from 0.8 M to 1.2 M and the residence time from 1.6 min to 5.6 min. However, further increase to 2 M did not improve the yield. On the other hand, further increase of residence time to 10.34 min positively affected the antibody recovery rate. In conclusion, optimal conditions were determined to be 1.2 M ammonium sulfate and the residence time of at least 10 min. Optimising the mentioned conditions improved the yield from 12% to 61% (Table 1).

3.2. Monomericity of purified IgE

The monomericity of anti-OVA IgE antibody purified under four different conditions (Table 1) was analysed using UPLC-SEC (Fig. 1A–D). Consistent peak patterns were observed across samples with the main peak retention time around 3.4 min and a monomericity ranging between 54 and 61%, while the aggregates levels were under 5.2% in most samples.

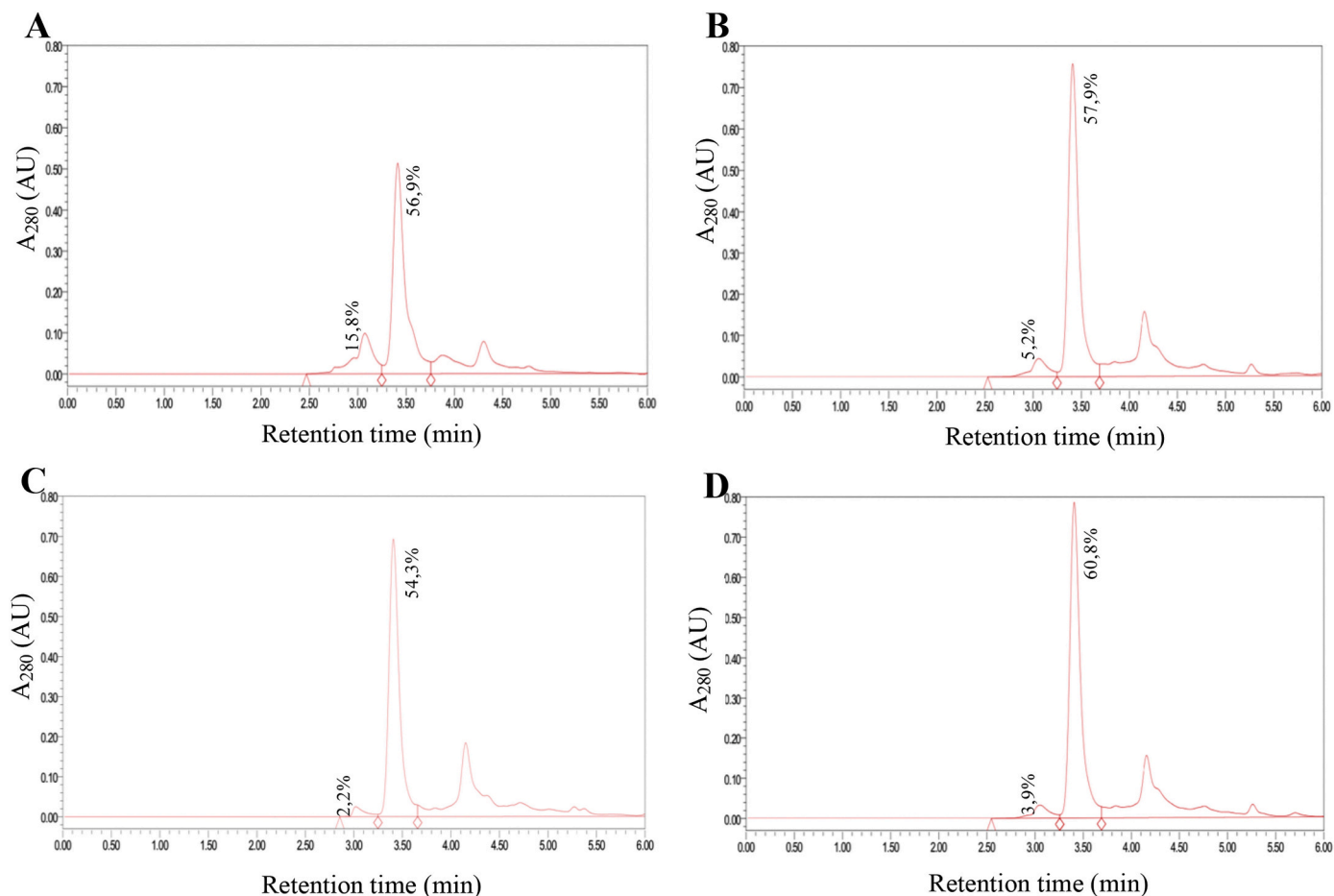
3.3. Scaled up purification of IgE antibodies produced by hybridoma or in a recombinant production system

Once the purification conditions were optimised, larger scale IgE purification was established. As starting material, 770 mL of the same 2C6 hybridoma supernatant was used. A bigger column with diameter of 1 cm was packed to a height of 19.1 cm and residence time of 17.65 min. A recovery rate of 59.3% (Table 2) and a monomericity of 73% (Fig. 2A) were achieved. In order to obtain even higher purity, preparative size-exclusion chromatography was performed (prep SEC). To this end, samples were eluted in 2 mL fractions and six fractions at the borders of the main peak (three in front and three in back) were analysed by UPLC-SEC (Fig. 2B). After pooling the monomeric fractions (fractions 23–31), a monomericity of 98.8% was achieved (Fig. 2C). 30.4 mg of highly pure IgE antibody was obtained as final product, constituting an overall recovery rate of 31% (Table 2).

In addition, TIC purification method was tested for anti-Thy1.1 IgE antibodies recombinantly produced in FreeStyle293 cells. To this end, 300 mL of FreeStyle293 cell supernatant containing an estimated concentration of about 222 mg/L IgE (Fig. S1B) were loaded on the $1\text{ cm} \times 20.9\text{ cm}$ column. The primary eluted sample showed a similar peak pattern as samples obtained from hybridoma, yielding a recovery rate of 65% and a monomericity of 55% (Fig. 2D). After prepSEC, the monomericity was improved to 97.7% (Fig. 2E), yielding an overall recovery rate of 27% (Table 2).

Table 1
Purification optimisation and IgE recovery.

Supernatant volume	Column length	Column diameter	(NH ₄) ₂ SO ₄ conc.	Column loading flow rate	Residence time	Recovered Ab	Estimated yield
50 mL	4.2 cm	0.5 cm	0.8 M	158 cm/h	1.6 min	0.76 mg	12%
25 mL	15.8 cm	0.5 cm	1.2 M	170 cm/h	5.6 min	1.44 mg	45%
25 mL	15.8 cm	0.5 cm	2 M	170 cm/h	5.6 min	1.04 mg	32.4%
25 mL	15.8 cm	0.5 cm	1.2 M	91.7 cm/h	10.34 min	1.96 mg	61%

**Fig. 1.** UPLC-SEC profiles of IgE samples purified by thiophilic chromatography under different conditions. Conditions used during TIC: (A) 0.8 M (NH₄)₂SO₄, residence time 1.6 min; (B) 1.2 M (NH₄)₂SO₄, residence time 5.6 min; (C) 2 M (NH₄)₂SO₄, residence time 5.6 min; (D) 1.2 M (NH₄)₂SO₄, residence time 10.3 min. Percentages of aggregates and monomeric antibodies are shown. Representative results are shown for two independent experiments.**Table 2**
Recovery rates of purified IgE antibodies produced by hybridoma or recombinantly.

Cell source	Step	Sample description	Volume [mL]	A280 (protein conc.)	IgE conc. [mg/mL]	Total IgE [mg]	Yield [%]
hybridoma	Supernatant	Anti-OVA IgE	770		128 (ELISA)	98,56	100
	Thiophilic resin	Purified IgE (elution)	17	5,51	3,44	58,5	59,3
	Prep SEC	Final product	16	3,04	1,9	30,4	30,8
FreeStyle293	Supernatant	Anti-Thy1.1 IgE	300		222 (ELISA)	66,6	100
	Thiophilic resin	Purified IgE (elution)	22	3,13	1,95	42,9	64,4
	Prep SEC	Final product	19	1,5	0,94	17,86	26,8

3.4. Purified IgE antibodies reach high purity and show expected molecular weight and conserved antigen binding activity

Molecular weight of purified antibodies was assessed by SDS-PAGE analysis. Anti-Thy1.1 IgG1 antibody was included for comparison purposes. Under reducing conditions, bands corresponding to heavy (about 95 kDa for IgE and 55 kDa for IgG₁) and light chains (28–30 kDa) were detected (Fig. 3A). The molecular sizes are within expected range for IgE

antibodies (Williams et al., 2019). Under non-reducing conditions, both IgE antibodies showed the same molecular size, whereas IgG₁ antibody was slightly smaller (Fig. 3B). No free heavy or light chain were detected, indicating that they are properly assembled into complete antibodies. The purity of final IgE products was tested on CE-SDS. No fragmentation was observed and the purity of both IgE antibodies was above 99% (Fig. 3C,D). Antigen binding activity of the anti-OVA and anti-Thy1.1 IgE antibodies was preserved as shown by binding ELISA

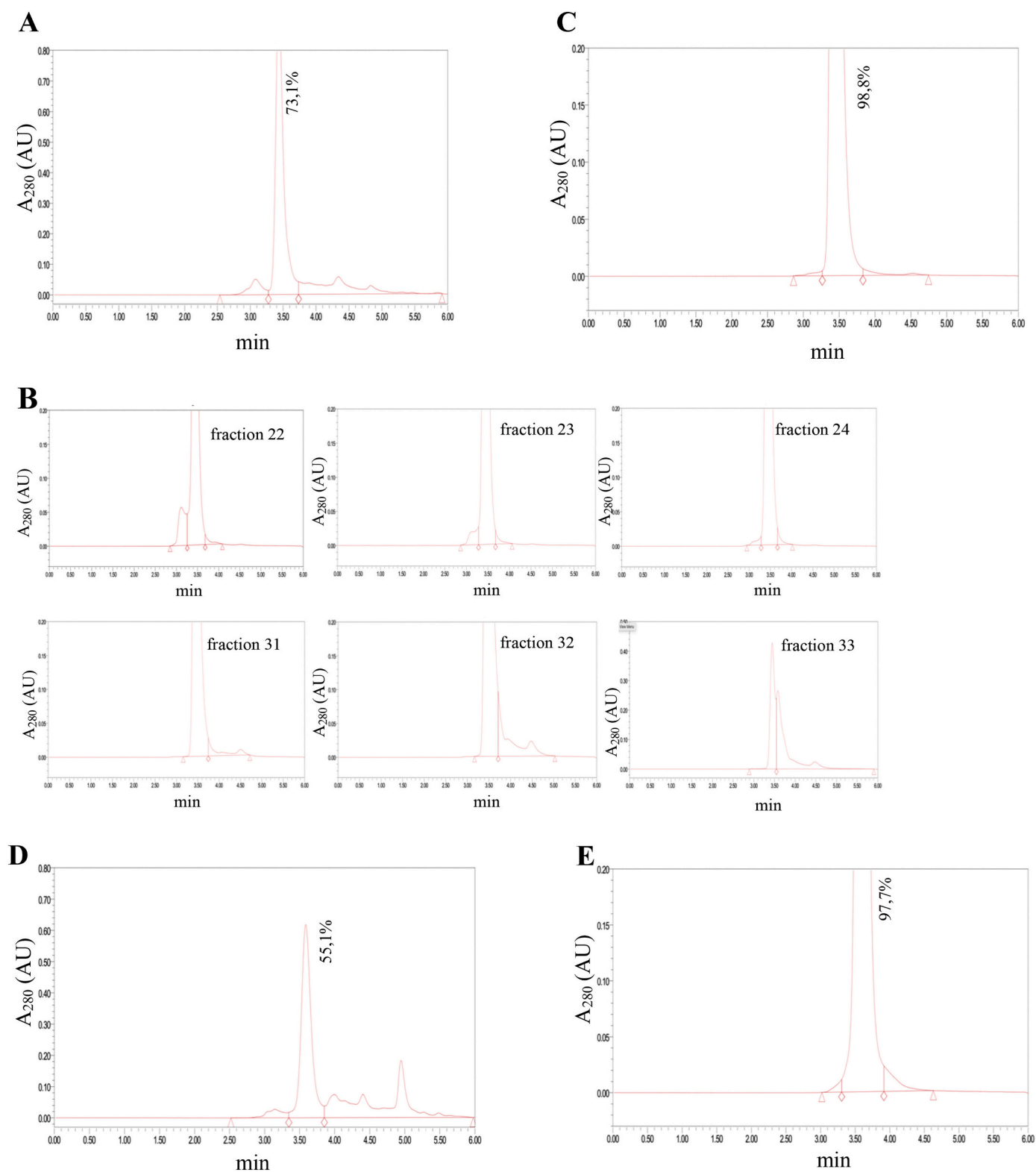


Fig. 2. Large scale purification of IgE antibodies produced by hybridoma and recombinant production system. UPLC-SEC profiles of (A) anti-OVA IgE antibody produced by 2C6 hybridoma and purified with thiophilic chromatography; (B) fractions of interest after prep SEC of purified anti-OVA IgE; (C) final anti-OVA IgE sample after the fractions 23-31 obtained by prep SEC were pooled together; (D) recombinantly produced anti-Thy1.1 IgE antibody and purified with thiophilic chromatography (before prep SEC); (E) recombinantly produced anti-Thy1.1 IgE antibody after prep SEC. Percentages of monomeric fractions are shown on chromatograms. Representative results are shown of at least two independent experiments.

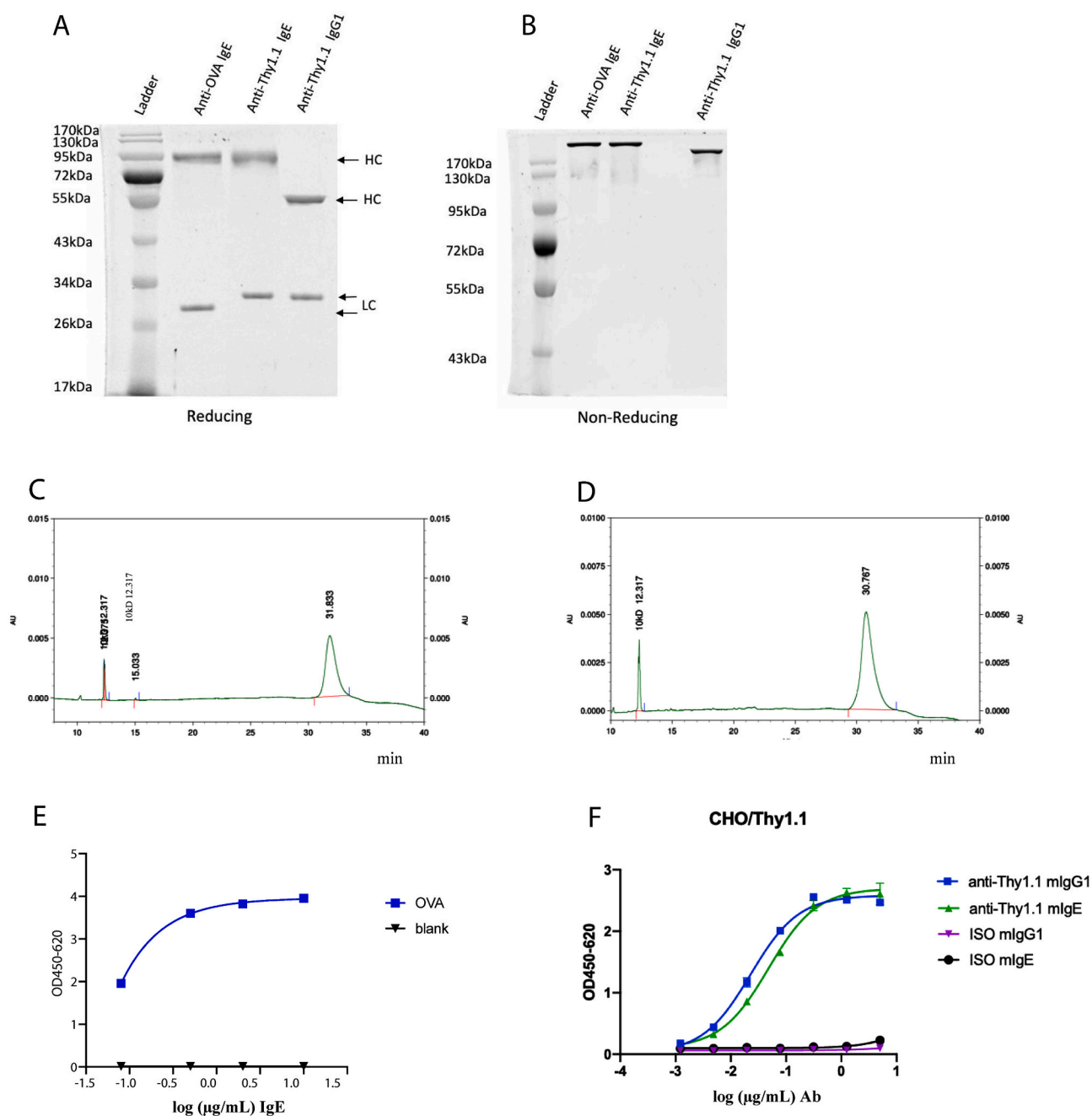


Fig. 3. Characterization of purified IgE antibodies. SDS-PAGE analysis of purified antibodies under (A) reducing and (B) non-reducing conditions; CE-SDS analysis of (C) anti-OVA IgE; (D) anti-Thy1.1 IgE; (E) binding ELISA of anti-OVA IgE; (F) binding ELISA of anti-Thy1.1 IgE and IgG1. Representative result shown of at least two independent experiments.

with chicken ovalbumin and Thy1.1 expressing CHO cells, respectively (Fig. 3E,F). No difference in binding activity was observed between anti-Thy1.1 IgE and IgG1, indicating that Ig format and purification method do not affect the binding to the antigen.

4. Discussion

With an increasing medical interest in IgE antibodies, there is also an increasing need for a cheap, simple and efficient purification method. Here, we report development and optimisation of IgE purification by thiophilic interaction chromatography.

UPLC-SEC data showed that the obtained antibodies were 54–73% monomeric, with the presence of several small molecular weight peaks. This indicates that the thiophilic resin might not be as specific for

immunoglobulins and that other proteins are being captured as well. Therefore, preparative SEC was required as additional purification step in order to improve monomericity and purity to >95%, which was set as the lower limit. If even higher monomericity is required, this could be achieved by an additional ion exchange step.

For IgE purification on industrial scale with large mass loading, preparative SEC may pose additional limitations as very large columns would be required. Therefore, more scalable methods, such as adsorption methods, may be more suitable for such applications. In addition, the need for an additional purification step such as preparative SEC might be avoided by further optimisation of the elution step of TIC. Here we performed isocratic elution, but longer gradient elution with decreasing ammonium sulfate concentration might better separate the impurities and provide an improved result and will, therefore, be the

subject of further studies.

The overall IgE recovery from initial supernatant was around 30%. However, it must be taken into consideration that ELISA was used for IgE quantification in the supernatant. This method has limited accuracy and can only provide an estimation of actual antibody concentration. The concentration of antibodies after purification was measured spectrophotometrically with high accuracy based on the absorbance at 280 nm. Further method optimisation in terms of column cross-section and loading settings may increase the recovery rate.

Furthermore, the purification method was tested on recombinantly produced IgE antibody. Recombinant antibody production offers a wide range of antibody designing possibilities and is becoming increasingly important. Our method showed consistent and reproducible results for both hybridoma produced and for recombinantly produced IgE antibodies. In addition, the conserved functionality of purified antibodies was confirmed in binding ELISA.

In conclusion, we have successfully purified murine IgE antibodies by thiophilic interaction chromatography. This method has a broad diversity and can be applied to any IgE irrespective of its specificity, light chain isotype or production system used. Although not tested with human IgE, given the nature of the interaction between the thiophilic resin and immunoglobulins, we expect this method to be applicable to human IgE as well. Potentially, thiophilic interaction chromatography could be given advantage over protein G or protein A based purification methods for other antibody isotypes as well, when gentle conditions in terms of pH are required.

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Declaration of Competing Interest

None.

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A human IgE bispecific antibody shows potent cytotoxic capacity mediated by monocytes

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The generation of bispecific antibodies (bsAbs) targeting two different antigens opens a new level of specificity and, compared to mAbs, improved clinical efficacy in cancer therapy. Currently, the different strategies for development of bsAbs primarily focus on IgG isotypes. Nevertheless, in comparison to IgG isotypes, IgE has been shown to offer superior tumor control in preclinical models. Therefore, in order to combine the promising potential of IgE molecules with increased target selectivity of bsAbs, we developed dual tumor-associated antigen-targeting bispecific human IgE antibodies. As proof of principle, we used two different pairing approaches - knobs-into-holes and leucine zipper-mediated pairing. Our data show that both strategies were highly efficient in driving bispecific IgE formation, with no undesired pairings observed. Bispecific IgE antibodies also showed a dose-dependent binding to their target antigens, and cell bridging experiments demonstrated simultaneous binding of two different antigens. As antibodies mediate a major part of their effector functions through interaction with Fc receptors (FcRs) expressed on immune cells, we confirmed FcεR binding by inducing *in vitro* mast cell degranulation and demonstrating *in vitro* and *in vivo* monocyte-mediated cytotoxicity against target antigen-expressing Chinese hamster ovary cells. Moreover, we demonstrated that the IgE bsAb construct was significantly more efficient in mediating antibody-dependent cell toxicity than its IgG1 counterpart. In conclusion, we describe the successful development of first bispecific IgE antibodies with superior antibody-dependent cell toxicity-mediated cell killing in comparison to IgG bispecific antibodies. These findings highlight the relevance of IgE-based bispecific antibodies for clinical application.

Antibody-based treatment in autoimmunity and cancer therapy has shown tremendous efficacy in clinical settings.

Consequently, biologicals such as mAb-based molecules represent one of the fastest growing classes of drugs in recent years (1). Among them, bispecific antibodies (bsAbs) have gained significant attention. The ability of one molecule to simultaneously target two different antigens opens a wide area of therapeutic applications, with cancer currently being the most targeted disease. Most of the bsAbs being developed as anticancer drugs can be classified into two groups: (1) immune cell engagers and (2) dual antigen targeting bsAbs (2). Immune cell engagers redirect the immune response toward the target cell by forming a bridge between an immune cell and a target cell. In addition, immune cell engagers induce the crosslinking of receptors on the immune cells. This crosslinking activates cytotoxic immune pathways, leading to target cell killing. In contrast, dual antigen targeting bsAbs target two different antigens on the target cell. Thus, the selectivity of the molecules for the target cell is increased and the risk of off-target side-effects decreased (3). In addition, if designed to interfere with two different signaling pathways in the tumor cell, dual tumor-associated antigens (TAAs) targeting bsAbs can bypass the development of treatment resistance (4). One prominent example of such a synergetic effect of bispecific antibodies is the recent clinical approval of amivantamab. This bsAb targeting epidermal growth factor receptor (EGFR) and cMet showed superior clinical efficacy to EGFR blockade monotherapy (5, 6).

Dual antigen targeting bsAbs can mediate their effector function *via* several pathways, such as complement-mediated cell lysis, antibody-dependent cell cytotoxicity (ADCC), or antibody-dependent cell phagocytosis. The isotype of the antibody can skew the immune reaction toward different effector functions, and hence, the selection of the isotype can be of critical importance for the efficacy of the treatment (7). Currently, all the mAbs with marketing approval are of the immunoglobulin (Ig) G class. However, other Ig isotypes, such as IgE and IgA, are also being explored, mainly as potential anticancer drugs. For instance, in addition to their well-known

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A human IgE bispecific antibody shows potent cytotoxicity

role in allergies mediated *via* mast cells (MCs), IgE antibodies can also bind to and, consequently, activate tumor-associated macrophages *via* FcεRI (8). Importantly, IgE mAbs have shown superior tumor control when directly compared to IgG in preclinical models (9–11). Furthermore, a phase I clinical trial evaluating an IgE-based molecule is currently showing promising results (12).

IgE isotype offers significant therapeutic advantages over IgG. For instance, IgE binds to its FcεRI with two orders of magnitude higher affinity than the IgG to its equivalent receptor (13). Therefore, IgE can stay bound to the immune cells expressing FcεRI, such as macrophages, monocytes, and basophils, even in the absence of antigen. Consequently, IgE shows extended tissue half-life (14). Moreover, IgE has no known inhibitory receptors (13), and contrary to IgG, which binds to the suppressive FcγRIIb, IgE only binds to activating FcRs. In preclinical models, mainly myeloid cells such as macrophages were identified as IgE effector cells (11, 15, 16), and IgE has been shown to mediate both ADCC *via* FcεRI and antibody-dependent cell phagocytosis *via* FcεRII (16). In addition to this direct effector function of IgE-based tumor treatment, tumors from rats treated with IgE tumor-targeting antibodies showed increased inflammation and macrophage skewing toward an M1-like phenotype (11). These findings suggest that, in addition to mediating superior direct cytotoxicity, IgE-based treatments can furthermore modify the tumor microenvironment in a favorable way (8).

In order to combine this rather promising potential of IgE molecules in cancer treatment with increased target selectivity, we developed dual TAAs targeting bispecific IgE antibodies. As proof of principle, we used two different approaches, one based on knobs-into-holes (KiH) and the other based on leucine zipper (LZ)-mediated molecule pairing. Here, we report the successful formation of bispecific IgE molecules with preserved antigen-binding properties and a fully functional Fc tail that mediates antigen-specific MC degranulation and *in vitro* and *in vivo* cytotoxicity.

Results

Design, expression, and purification of IgE bsAbs

Interspecies differences in FcεR expression between mice and human (17, 18) exclude the use of mice and murine IgE as an adequate model for the study of IgE biology. Thus, we decided to design human bsAbs. For this purpose, two different approaches were used: (1) KiH and (2) LZs. As a proof of concept, we used in both approaches one conventional antigen-binding fragment (Fab) and a single domain antibody (sdAb) (19, 20) (Fig. 1, B and C). Because the sdAb arm does not contain a light chain, it is smaller than a conventional heavy chain. Consequently, the Fab×sdAb-Fc format offers important advantages. Firstly, due to size differences between Fab and sdAb arm, evaluation of bsAb formation can be easily done by SDS-PAGE. Secondly, any accidental light chain–heavy chain mispairing is prevented (19). As a choice of model antigens, we relied on a well-established system in our research group comprised of a conventional antibody targeting mouse prostate-specific membrane antigen (PSMA), combined with a sdAb targeting the EGFR (19, 20).

KiH is a commonly used heterodimerization technology for IgG molecules (21, 22). Amino acids of constant heavy domains are engineered to form a knob in one heavy chain represented by a bulky amino acid, and a hole in an opposite heavy chain represented by a small amino acid. These mutations force the heterodimerization and formation of a bsAb. In human IgE molecules, the second heavy chain domain (CH2) is responsible for the dimerization of heavy chains and is, therefore, a target for KiH engineering. The use of human CH2 IgE domains with KiH mutations has been previously reported in an EFab Domain Substitution technology (23). The authors replaced CL-CH1 domains of one arm of a bispecific IgG molecule with CH2 IgE domains featuring KiH mutations in order to prevent light chain–heavy chain mispairing (Fig. 1A). The mutations described are S10I in one CH2 domain, with isoleucine forming a hydrophobic knob, and T121G in an opposite CH2 domain,

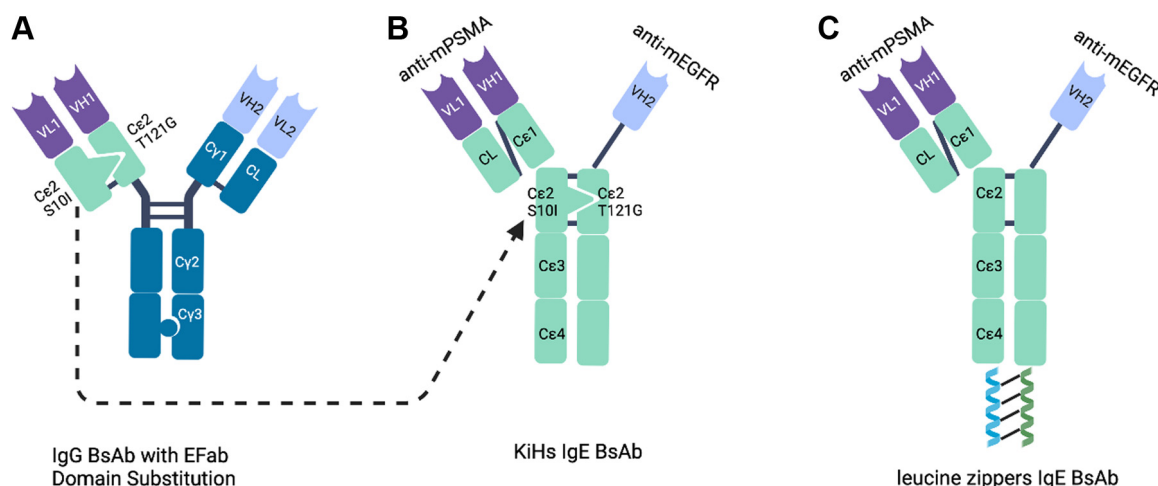


Figure 1. Design of bispecific IgE molecules. A, IgG bispecific antibody with Efab domain substitution in which CL and Cy1 domains in one Fab were replaced with Cε2 domains featuring KiH mutations. B, IgE bispecific with KiH mutations in Cε2 domains. C, IgE bispecific with leucine zippers. KiH, knobs-into-holes.

with glycine forming a hole. Here, we used the reported mutations in a whole IgE molecule to obtain a human bispecific IgE antibody (Fig. 1B). S10I point mutation was introduced in the PSMA arm, whereas T121G point mutation was introduced in the EGFR arm. *In silico* 3D modeling was used to assess the outcome of the potential interactions between the heavy chains carrying the mutations (Fig. 2). Once the expression vectors are transfected into cells, several different dimerizations could theoretically occur: EGFR T121G homodimer, EGFR T121G x PSMA S10I heterodimer, and PSMA S10I homodimer. For the EGFR T121G homodimer, the lack of the 'knob' mutation showed a gap in the interface between the CH2 domains (Ser10-

Gly121) in the 3D model (Fig. 2A). At the same time, a lack of the 'hole' mutation in the PSMA S10I homodimer results in the multiple clashing between the two chains with the distance less than 1.5 Å (Ile10-Thr121) (Fig. 2C). Only the EGFR T121G x PSMA S10I heterodimer model showed an excellent KiH fit (Ile10-Gly121) (Fig. 2B). Therefore, the 3D modeling suggested that the EGFR T121G x PSMA S10I heterodimer would preferentially be formed over the EGFR T121G homodimer and PSMA S10I homodimer.

In a parallel approach, we introduced fos and jun peptide-based LZ pairs on the C terminus of the two different heavy chains. Fos and jun form a LZ complex (24), which we

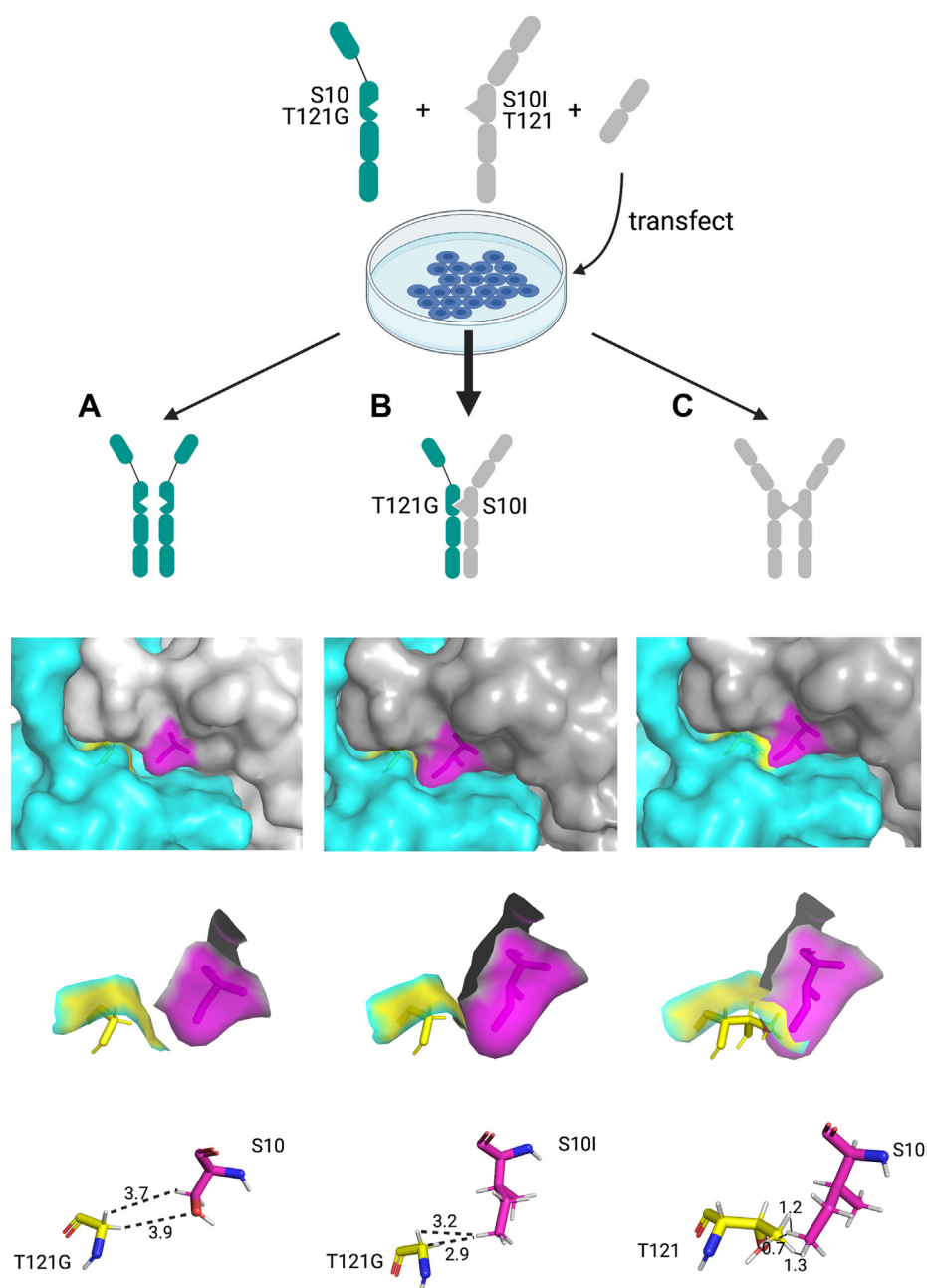


Figure 2. *In silico* 3D modeling of potential pairings of IgE heavy chains featuring KiH mutations. A, αEGFR T121G homodimer. B, αEGFR T121G x αPSMA S10I heterodimer. C, αPSMA S10I homodimer. Two heavy chains in gray and cyan; S10I in magenta, T121G in yellow. The dashed lines indicate the distance measurements in angstrom (Å). KiH, knobs-into-holes; PSMA, prostate-specific membrane antigen.

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hypothesized would drive the heterodimerization of the two heavy chains (Fig. 1C).

All IgE bsAb constructs and monospecific controls were recombinantly produced. Following expression, hetero-complexes and anti-EGFR monospecific antibody were affinity purified based on the His-tag present in the EGFR arm, while anti-PSMA IgE was affinity purified using a Strep-Tactin column. The purified antibody complexes were then analyzed by SDS-PAGE to check for bispecific IgE formation (Fig. 3). As expected, under reducing conditions, one band at ~70 kDa corresponding to the heavy chain was detected for monospecific anti-EGFR antibody; two bands at ~90 kDa and ~30 kDa, corresponding to heavy and light chain, respectively, were detected for monospecific anti-PSMA antibody; and three bands at ~90 kDa, ~70 kDa, and ~30 kDa corresponding to anti-PSMA heavy chain, anti-EGFR heavy chain, and anti-PSMA light chain, respectively, were detected for bispecific anti-PSMAxEGFR antibodies obtained by both KiH and LZ strategies. Under nonreducing conditions, expected differences in size were detected, with monospecific anti-EGFR IgE being the smallest (~130 kDa), followed by bispecific IgE antibody (~170 kDa), and finally, monospecific anti-PSMA IgE (~210 kDa). Of note, some aggregates consistently formed during purification of anti-EGFR parental antibodies and, to less extent, with bispecifics (Fig. 3B). The aggregate formation can be assumed to be induced by the presence of imidazole in the elution buffer (25), as we usually do not experience such aggregates with this antibody and as the anti-PSMA parental antibody purified by Strep-tag affinity chromatography was free of aggregates. Therefore, for future bsAb purifications, a

His-tag independent approach might want to be considered. Furthermore, in one batch, an additional band at ~220 kDa was observed for the LZ-containing bispecific IgE. As this band was not present in other production batches (data not shown), we assume it to be a batch-specific impurity. Nevertheless, taken together, our SDS-PAGE-based analysis of purified antibodies clearly indicated the successful formation of the bispecific IgE antibody by both KiH and LZ strategies.

To evaluate the efficacy of bispecific IgE formation, we checked for the presence of bands derived from potential unwanted pairings. After the first bispecific IgE purification step based on His-tag present in the EGFR arm, no EGFR homodimer was observed on SDS-PAGE. Next, the supernatant went through a second purification step in a Strep-tag column in order to recover the potentially formed PSMA homodimer. Also, after this second step, we detected no additional products (data not shown), indicating that the bispecific IgE complexes were formed with a high-efficiency rate, as predicted by 3D modeling (Fig. 2).

Binding activity of bispecific IgE

Next, we wanted to confirm that the formed bsAbs retained their antigen-binding properties. To this end, single-arm binding of the produced anti-EGFRxPSMA bispecific IgE antibody was tested using stably transfected Chinese hamster ovary (CHO)/mPSMA and CHO/mEGFR cell lines by flow cytometry. Respective monospecific parental antibodies were used as a positive control. The monovalent-binding activity of anti-EGFRxPSMA bispecific KiH and LZ IgE antibodies to both CHO/mEGFR cells and CHO/mPSMA cells was

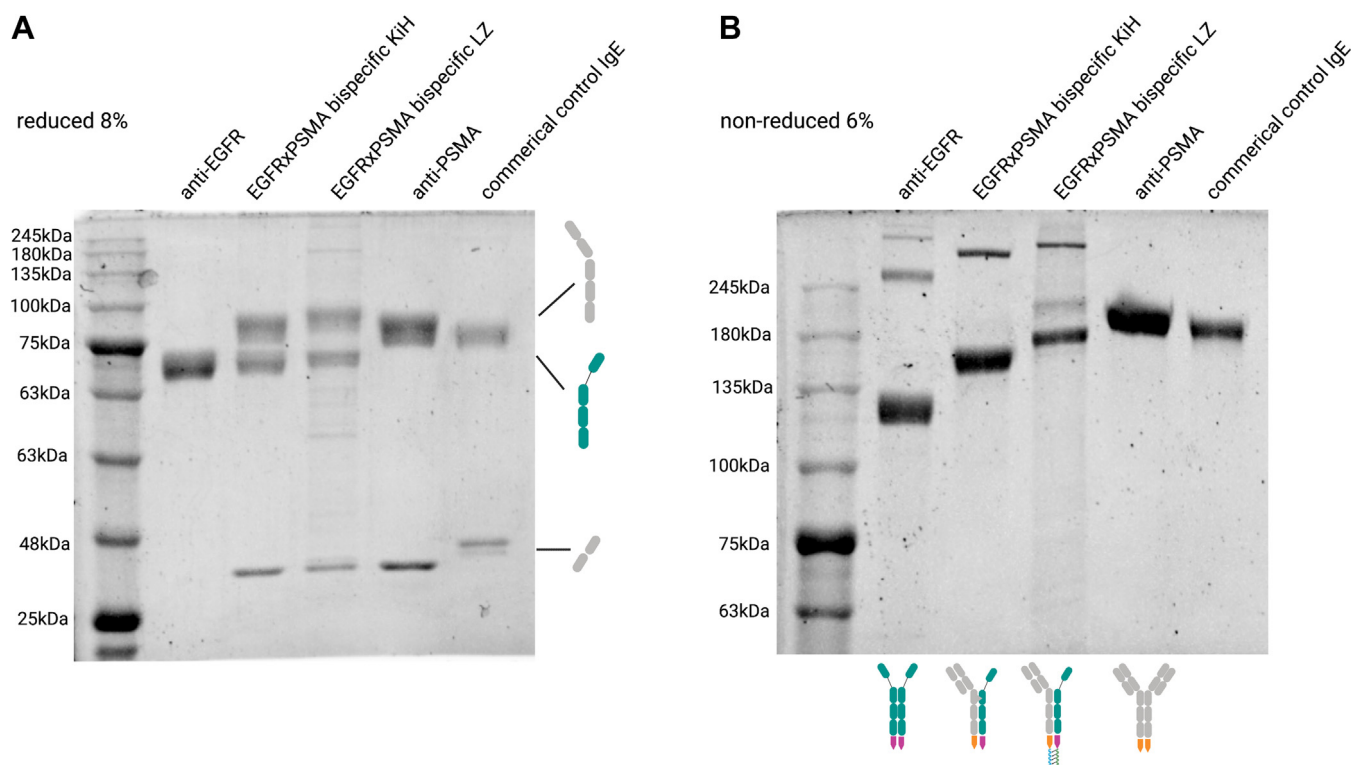


Figure 3. SDS-PAGE analysis of purified IgE monospecific and bispecific antibodies shows correct formation of bispecific IgE antibodies. The purified antibodies were analyzed under (A) reducing and (B) nonreducing conditions.

comparable to the bivalent binding of monospecific anti-EGFR and anti-PSMA IgE antibodies, respectively (Fig. 4, A and B and Table 1). The binding of bsAb to both antigens was dose dependent. As a negative control, we used CHO.K1 cells and did not observe any binding, as expected. These results show that the specificity of both arms within the bispecific IgE antibody is preserved.

Next, the simultaneous binding to both target antigens was tested in a cell-bridging experiment. In short, e670-labeled

CHO/mPSMA cells were incubated with anti-EGFRxPSMA bispecific IgE antibodies, after which the unbound antibody was washed and e450-labeled CHO/mEGFR cells were added. Double-stained cell clusters were detected by flow cytometry, typically reaching levels of a quarter to a third of all cells measured (Fig. 4C).

In summary, these data show that the bispecific IgE antibody retained the binding activity toward its target antigens and its capacity for simultaneous binding of both antigens.

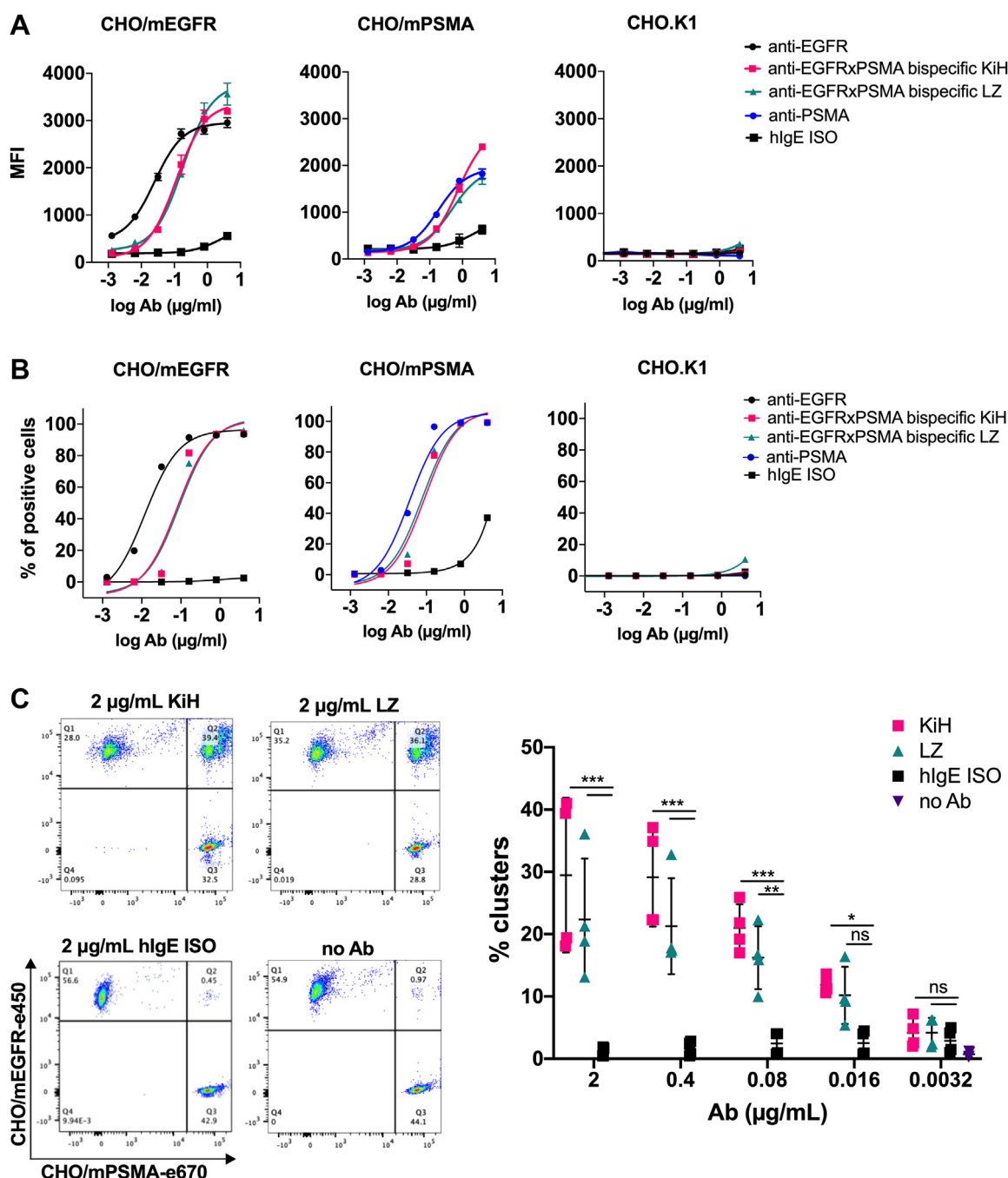


Figure 4. Flow cytometry evaluation of antigen-binding properties of bispecific IgE antibodies. Single-arm binding to CHO/mEGFR, CHO/mPSMA, and CHO.K1 cells in a dose-dependent manner represented by (A) MFI values and (B) percentage of positive cells. Each data point is the mean $\pm$ SD of duplicates. C, bsAbs-induced cellular clustering by simultaneous binding to CHO/mEGFR and CHO/mPSMA cells. Pooled data from two independent experiments performed in duplicates are shown. Statistical significance was determined with two-way ANOVA with Dunnett's multiple comparison test (*** $p < 0.0001$, ** $p = 0.002$, * $p = 0.04$). CHO, Chinese hamster ovary; MFI, mean fluorescence intensity; PSMA, prostate-specific membrane antigen.

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Table 1

EC₅₀ values of tested antibodies (cell-binding assay)

EC ₅₀ (μg/ml)	aEGFR IgE	Bispecific KiH	Bispecific LZ	aPSMA IgE
CHO/ mEGFR	0.025	0.11	0.18	/
CHO/ mPSMA	/	0.73	0.48	0.18

The functionality of the Fc tail is preserved in the bispecific IgE molecule

In order to confirm that the introduced KiH mutations or LZ do not affect the FcεR binding, the ability to induce MC degranulation was tested. To this end, human MCs were coated with IgE antibodies, after which the crosslinking was induced with anti-IgE antibody, and degranulation was estimated as the percentage of β-hexosaminidase (β-hex) release. Anti-EGFRxPSMA bispecific IgE induced a similar level of dose-dependent MC degranulation as monospecific anti-EGFR and anti-PSMA IgE controls (Fig. S3). These results indicate that the binding of the bispecific IgE antibody to FcεR was retained.

Furthermore, we tested whether the bispecific IgE induces MC degranulation once it encounters the antigen-expressing cells. In this setup, human MCs coated with IgE were added to plated CHO/mPSMA or CHO/mEGFR cells, and β-hex release was measured. To show a dose-dependent effect, antigen-specific IgEs were mixed with unspecific commercial IgE (AG30P) in different ratios but maintaining the total MC coating concentration at 2 μg/ml. Bispecific IgE KiH and LZ molecules showed dose-dependent MC degranulation, reaching the highest levels of ~40% to 50% at 0.4 μg/ml (Fig. 5, A and B). Results of the β-hex release were mirrored by IL-8 levels measured in the supernatant (Fig. S4), further confirming MC activation. Of note, anti-mPSMA IgE induced a somewhat higher level of MC degranulation upon binding to CHO/mPSMA cells (Fig. 5A). This could be expected as bispecific IgE molecules bind to PSMA with a single arm and, therefore, would be less efficient in inducing FcεR crosslinking that is required for MC degranulation. On the other

hand, anti-EGFR IgE mediated MC degranulation was considerably lower (~20%) in all tested conditions (Fig. 5, A and B). When anti-EGFR IgE sensitized MC was degranulated with TX100 as a positive control, the β-hexosaminidase release was also 50% lower when compared to other antibodies (data not shown). Aggregates present in the anti-EGFR IgE may likely have induced such effects (Fig. 3B). We observed elongated MC after the sensitization step with this IgE, indicating that the aggregates directly activated a part of MC (Fig. S5). Thus, during the sensitization, some level of activation may already have occurred, making the activated part of MC unresponsive to the crosslinking in the following step and resulting in an overall lower degranulation extent.

In summary, bispecific IgE antibodies obtained by both KiH and LZ technologies successfully induced MC degranulation upon antigen binding, indicating that both Fc-tails are functional.

Bispecific IgE mediates cytotoxicity in vitro and in vivo

Next, to fully confirm the functionality of IgE bsAb, we tested whether it is capable of inducing cytotoxicity. Thus, we performed *in vitro* ADCC assays targeting CHO/mPSMA_-mEGFR cells, using purified human peripheral blood CD11b⁺ cells as effector cells. In this set of experiments, we directly compared IgE bsAb made by KiH strategy to IgG1 bsAb with the same specificity. Our data show that IgE bsAb induced cell death at a range of ~40%, which was consistently significantly higher than IgG1 bsAb, inducing around 25% cell death (Fig. 6A). No unspecific ADCC effect was observed against CHO.K1 cells used as a control (Fig. 6B). The level of aggregates present in bispecific IgE samples was not high enough to induce MC activation in the MC degranulation experiments (Fig. 5). Therefore, it is highly unlikely that the low amount of aggregates present in IgE bsAb preparations may have caused any biological effect in the ADCC experiments. Such an assumption is further supported by the absence of killing of CHO.K1 cells (Fig. 6B). Thus, taken together, our data demonstrate that IgE bsAb-mediated ADCC is more efficient

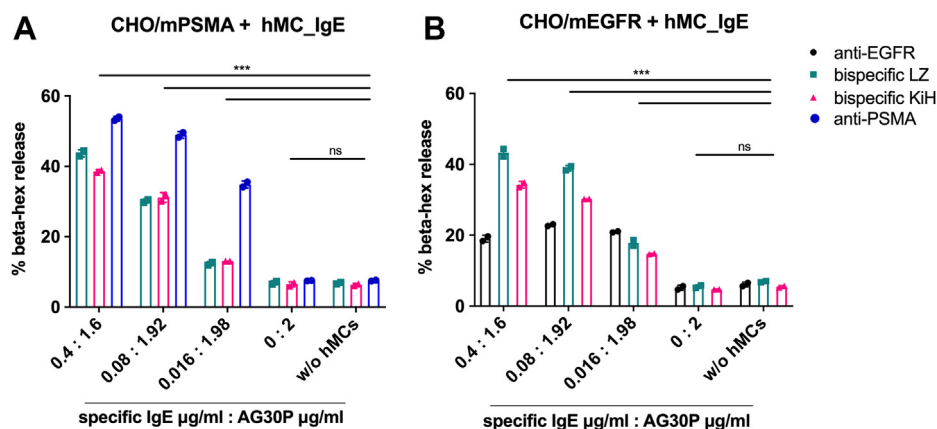


Figure 5. β-hexosaminidase release after mast cell degranulation induced by bispecific IgE antibodies. Human mast cells were sensitized with indicated mixtures of specific IgEs and AG30P unrelated IgE and added to plated (A) CHO/mPSMA cells or (B) CHO/mEGFR cells. Mean ± SD of duplicates is shown. Statistical significance was determined with two-way ANOVA with Dunnett's multiple comparison test ($p < 0.0001$). CHO, Chinese hamster ovary; PSMA, prostate-specific membrane antigen.

than that of its IgG1 bsAb homolog, indicating a greater cytotoxic potential of IgE isotype when CD11b⁺ cells are provided as effector cells.

Finally, we studied the antibody-mediated *in vivo* cell killing in a murine model. To this end, Rag2^{-/-}γc^{-/-} immunodeficient mice were injected with 5-chloromethylfluorescein diacetate (CMFDA)-labeled CHO/mPSMA_EGFR cells, human CD11b⁺ effector cells, and bispecific IgE. As human IgG1 is crossreactive with murine Fcγ receptors and has been reported to induce tumor cell killing by activating murine effector cells (26), we omitted a direct control of IgG-mediated *in vivo* killing. After 24 h, transferred cells were recovered by peritoneal lavage, and the number of remaining target cells (CHO/mPSMA_EGFR) and CD11b⁺ cells were assessed by flow cytometry. The number of recovered live target cells was lower in IgE-treated mice when compared to the control group (Fig. 6C). In contrast, the recovered CD11b⁺ effector cell population remained the same in the mouse groups that received IgE bsAb or not (Fig. 6D). In order to internally normalize the number of recovered cells, we used the ratio

between the numbers of live target cells and live CD11b⁺ effector cells. Tumour/effector ratio was significantly lower in IgE-treated mice when compared to the control group (Fig. 6E), clearly demonstrating IgE induced the cell killing *in vivo*.

In conclusion, IgE bsAb exhibited greater *in vitro* cytotoxic capacity than its IgG counterpart and was able to mediate *in vivo* target cell killing.

Discussion

The introduction of mAbs in clinical settings fundamentally changed the current way cancer is treated, achieving success in previously untreatable types of cancer. Furthermore, the most recent clinical approval of amivantamab (6), a bsAb targeting the EGFR and cMet, has made apparent that the dual TAAs targeting bsAbs can show improved efficacy over monotherapy.

However, ever since it became evident that the Fc part plays an important role in the antibody efficacy, a tremendous effort has been made to further optimize IgG molecules, the

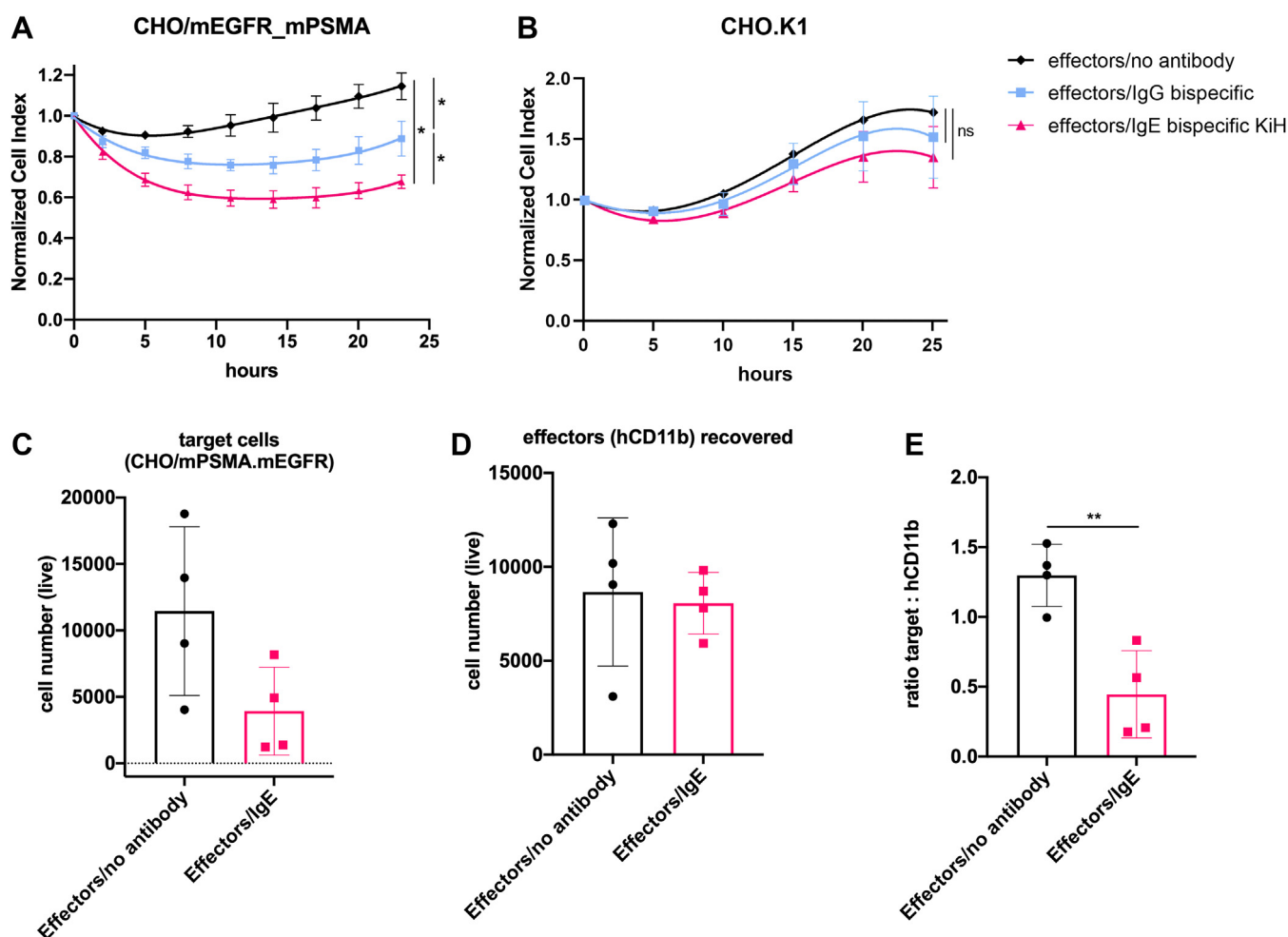


Figure 6. Bispecific IgE induces cytotoxicity *in vitro* and *in vivo*. Real-time ADCC assay against (A) CHO/mPSMA.mEGFR cells and (B) CHO.K1 cells and hCD11b⁺ cells as effectors. Representative data of two independent experiments performed in duplicates are shown. Normalization was done ~5 h after the effector cell addition. Statistical significance was determined using an extra sum-of-squares F test ($p < 0.0001$) (C) Rag2^{-/-}γc^{-/-} mice were intraperitoneally injected with CMFDA-labeled CHO/mPSMA.mEGFR target cells, indicated antibodies and hCD11b⁺ cells as effectors. Recovered number of live target cells (C) and hCD11b⁺ cells (D) after 24 h is shown. E, ratio between recovered number of live target cells and hCD11b⁺ cells after 24 h. Statistical significance was determined by unpaired two-tailed *t* test ($p = 0.0044$). (n = 4). CHO, Chinese hamster ovary; PSMA, prostate-specific membrane antigen.

commonly used antibody isotype. The Fc engineering focused on various aspects, including: increasing the half-life by improving the binding to FcRn, optimizing the activation *versus* inhibition (A/I) ratio by reducing the interaction of the Fc-part with inhibitory FcγRIIb and increasing the binding to activatory FcγRs, and optimizing complement activation (7). Switching to the IgE isotype solves two of the mentioned aspects. IgE is characterized by extended tissue half-life and lacks binding to inhibitory Fc receptors. In addition, IgE has been shown to offer superior macrophage tumor infiltration and the ability to re-educate them toward the M1 phenotype, which resulted in better tumor control when compared to IgG (8). Given the promising role of IgE-based cancer therapeutics, the bispecific IgE antibody represents a very attractive novel antibody format that would combine the advantages of IgE isotype with increased tumor specificity characteristic for bispecific molecules. The work presented here describes the successful generation of IgE bsAbs by two different approaches—KiH and LZ-mediated pairing.

We made use of the currently still unconventional Fab $\times$ s-DAb-Fc format in both strategies (19). This format allowed us to clearly demonstrate by SDS-PAGE analysis that IgE bsAb formation by both KiH and LZ approaches was highly successful and that unwanted pairings were absent. These findings suggest that described strategies efficiently drive IgE heavy chain heterodimerization. In order to avoid light and heavy chain mismatching in more conventional bsAb formats, where two heavy chains and two light chains are present, some alternative solutions have been described for IgG-based bsAb, which could be adapted for bispecific IgE molecules as well (27, 28).

Our data show that the bispecific IgE antibodies preserved antigen-binding properties and antigen specificity of both arms in addition to the fully functional Fc part, as they induced MC degranulation upon antigen binding. An important finding of this study is the superior ADCC effect of bispecific IgE compared to its IgG bispecific homolog with the exact variable domains and, hence, identical antigen-binding properties. Here, human CD11b⁺ cells, mainly represented by monocytes, were provided as effector cells, further supporting previously published findings of superior macrophage activation by IgE isotype, including the subsets likely found in the tumor microenvironment (8, 11, 29). Macrophages are the most abundant type of immune cells in the tumor environment (30). Thus, our findings strongly suggest that IgE isotype-based antibodies, including bispecifics, can be therapeutically superior to those with an IgG Fc-part.

As IgE antibodies not only bind to macrophages but also to MCs and basophils and are well known to induce allergic reactions, the aspect of anaphylaxis has been a great concern for the use of IgE mAbs in tumor therapy. However, preclinical (31–33) and initial clinical safety data (12) with tumor antigens targeting monospecific IgE antibodies did not suggest any increased risks, and the safety profile appeared satisfactory. Systemic allergic reactions can be a risk if IgE antibodies encounter circulating tumor cells expressing the target antigen. As circulating tumor cells are generally present in only minute quantities, such a reaction appears highly unlikely for both monospecific and

bispecific IgE antibodies. Furthermore, as bsAbs can substantially increase target selectivity, we would like to argue that the bispecific IgE format may even further improve the safety of IgE therapy and reduce the risk of systemic off-site reactions.

Furthermore, as with all proof-of-concept studies, our work also has certain limitations. For instance, the antigens and model systems chosen for this study were somewhat artificial. However, the described technology for IgE bispecific generation can now be used to target physiologically more relevant tumor antigens that would allow us to test the *in vivo* effect in immunocompetent human IgE $\times$ R-transgenic preclinical models and, consecutively, in a clinical setting.

In conclusion, we report the generation of fully functional tumor targeting human IgE bsAb with preserved antigen binding and superior cytotoxic capacity than its IgG homolog. The described IgE bispecific formats open a wide area for further IgE exploitation as cancer therapeutics by arming the potent IgE molecules with increased tumor specificity.

Experimental procedures

Cell culture

HEK293T cells were grown in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 50 μ M 2-mercaptoethanol (Gibco). CHO.K1 cells (ATCC; catalog no.: # CCL-61) were grown in Advanced Dulbecco's modified Eagle's medium/Ham's F-12, (DMEM/F-12; Gibco), supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. Culture conditions were maintained at 37 °C in a humidified atmosphere containing 7% CO₂.

FreeStyle 293 cells (Invitrogen) were grown in FreeStyle 293 Expression medium (Invitrogen) on a CO₂ resistant shaker (Thermo Scientific) at 37 °C, 8% CO₂, and 120 rpm.

CHO/mPSMA and CHO/mEGFR stable transfected cell lines were previously developed within our research group (19). They were grown in Advanced DMEM/F12 medium supplemented with 5% heat-inactivated FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, and 0.8 mg/ml G418 (Gibco). Culture conditions were maintained at 37 °C in a humidified atmosphere containing 7% CO₂.

CHO/mEGFR_mPSMA cell line was obtained by stably transfecting CHO/mEGFR with pcDNA3.1/hygro+.mPSMA as described previously (19). The stable clone was selected after 10 to 14 days of growth under hygromycin (0.6 mg/ml) and geneticin (0.8 mg/ml) pressure, after which single-cell sorting was performed on a FACSMelody (BD). The expression was regularly monitored by flow cytometry with an anti-mPSMA antibody (Sam103 (19)), and the selected clone showed stable PSMA expression for more than a month.

For ADCC assay, the CHO/mEGFR_mPSMA cells were grown in DMEM/F12 (Gibco; 11320033) supplemented with 5% heat-inactivated FBS, 1% penicillin/streptomycin, 0.8 mg/ml geneticin, and 0.6 mg/ml hygromycin. Culture conditions were maintained at 37 °C in a humidified atmosphere containing 7% CO₂.

Design of vectors

The anti-mEGFR sdAb (RR359) has been described before (20), and the amino acid sequence of the anti-mPSMA antibody (Sam103) was obtained from patent US20170342169A1. The sequence of IgE constant heavy chain was obtained from the UniProt database (ID: P01854). The sdAb (anti-EGFR) was linked to CH2-CH4 IgE constant domains *via* a partial llama linker (EPKTPKPQPQPQPQP).

Signal peptides were introduced at the N terminus. For KiH technology, an S10I point mutation was introduced in the CH2 domain of the PSMA arm, whereas T121G point mutation was introduced in the CH2 domain of the EGFR arm. At the C terminus of PSMA and EGFR arms, Strep-tag and His-tag were introduced, respectively. The tags were followed by a stop codon, thrombin cleavage site, and fos or jun LZ sequences, respectively. An additional thrombin cleavage site was introduced upstream fos/jun sequences so that the LZ can be removed by thrombin digestion after antibody purification, if needed. However, for this set of experiments, the LZ had not been cleaved off. These initial mAb expression vectors were constructed by *de novo* synthesis (Biomatik). Some of the expression vectors (for parental monospecific antibodies and anti-EGFR-jun) were then obtained by Q5 site-directed mutagenesis in order to reverse KiH mutations and delete stop codons. The success of site-directed mutagenesis was confirmed by Sanger sequencing (Genewiz). For all antibodies, pcDNA3.1(+) was used as an expression vector. A schematic representation of IgE antibody constructs is given in Fig. S1. Bispecific human IgG1 featured the same variable chains as IgE. It was obtained by previously described KiH mutations (T366S, L368A, and Y407V point mutations were introduced to the CH3 region of the EGFR arm, a T366W point mutation was introduced to the CH3 region of the PSMA arm). Amino acid sequences of all antibodies are given in Table S1.

In silico modeling

In silico modeling was done using PyMOL Molecular Graphics System (version 2.3.3, Schrödinger, LLC). Mutant models were generated using the reported crystal structure of IgE Fc (Protein Data Bank entry 2WQR) as a starting model. Water molecules were removed, adaptive Poisson–Boltzmann solver electrostatics calculations performed, and indicated amino acids were mutated. For the mutation T121G, there are no rotamers for Glycine. For the mutation S10I, we used isoleucine rotamer with highest frequency suggested by PyMOL (60%).

Recombinant antibody production

Bispecific IgE antibodies and anti-EGFR IgE antibody were recombinantly produced in HEK293T cells. Briefly, pcDNA3.1(+) expression vectors encoding corresponding heavy and light chains (1:1 ratio for monospecific antibody, 1:1:1 ratio for bsAbs) were transfected into HEK293T cells (80% confluent) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. After 6 h, the cells were gently washed with PBS and fresh FreeStyle 293 Expression Medium (Invitrogen) was added. The cells were incubated for

7 days at 37 °C in a humidified atmosphere containing 7% CO₂. On day 7 post-transfection, the cell suspension was collected and centrifuged for 15 min at 2500g. The supernatants were filtered over a 0.22 µm filter and stored at 4 °C.

Anti-PSMA IgE antibody and bispecific IgG1 antibody were recombinantly produced in FreeStyle293 cells. Briefly, the cells were transfected with pcDNA3.1(+) expression vectors encoding corresponding heavy and light chains (1:1:1 ratio for bispecific antibody), using 293fectin reagent (Invitrogen) according to the manufacturer's recommendation. The cells were incubated for 7 days at 37 °C, with 8% CO₂ at 120 rpm. On day 7 post-transfection, the cell suspension was collected and centrifuged for 15 min at 2500g. The supernatants were filtered over a 0.22 µm filter and stored at 4 °C.

His-tag-based antibody purification

The supernatant containing the IgE antibodies was mixed with a predetermined amount of Ni Sepharose excel (Cytiva) and rotated overnight at 4 °C. Following overnight capturing, the resin was washed once with PBS, and the bound antibody was purified from the resin by two-step elution with 50 mM imidazole pH 7.5, followed by 1 M imidazole pH 7.5. The antibody was rebuffed to PBS using either Zeba Spin (Thermo Scientific) or PD-10 (Cytiva) desalting columns according to the manufacturer's instructions.

Strep-tag-based antibody purification

To neutralize the biotin present in the FreeStyle293 medium, which can interfere with the Strep-Tactin column, a BioLock solution (Iba LifeSciences) was added to the supernatant containing IgE antibodies according to the manufacturer's recommendation. Next, the supernatant was loaded onto a manually packed Strep-Tactin column (CV = 2 ml) previously equilibrated in 100 mM Tris–HCl pH 8.0, 150 mM NaCl, and 1 mM EDTA. The antibody was eluted with a buffer (100 mM Tris–HCl, pH 8.0/150 mM NaCl, and 1 mM EDTA) containing 2.5 mM desthiobiotin into 1 ml fractions. The fractions were analyzed by SDS-PAGE, and the ones that contained the antibody were combined. The antibody was rebuffed to PBS using either Zeba Spin or PD-10 desalting columns according to the manufacturer's instructions.

IgG purification

Bispecific IgG antibody was purified as described before (19) by protein A affinity chromatography.

Antibody quantification

All antibodies were quantified using a NanoDrop spectrophotometer at 280 nm. Antibody concentration was calculated based on the Beer–Lambert Law, $A = \epsilon \cdot b \cdot c$, (A is the A_{280} absorbance, b is the path length, c is the analyte concentration, and ϵ is the wavelength-dependent molar absorptivity coefficient with units of $M^{-1} \text{ cm}^{-1}$). The coefficients were calculated using the ProtParam online tool based on the amino acid sequences.

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SDS-PAGE

One microgram of each antibody was diluted in Laemmli sample buffer (Bio-Rad) for analysis under nonreducing conditions or in Laemmli sample buffer containing 10% β -mercaptoethanol for analysis under reducing conditions. Samples were heated either at 95 °C for 5 min (reduced) or at 90 °C for 2 min (nonreduced). Samples and BLUeye Prestained Protein Ladder (Geneflow) were loaded to either 8% polyacrylamide gel (reduced samples) or 6% polyacrylamide gel (nonreduced samples). The gels were run at 70 V for 30 min, after which the voltage was increased to 120 V for 60 min. Gels were stained using GelCode Blue Safe Protein Stain (Thermo Scientific), fixed for 30 min in buffer containing 40% ethanol and 10% acetic acid, destained with distilled water, and scanned using ChemiDocTM Touch Imaging System (Bio-Rad).

Cell-binding assays by flow cytometry

CHO.K1, CHO/mEGFR, and CHO/mPSMA cells were detached and washed once with flow cytometry staining buffer (FACS buffer: PBS + 2% FBS). Indicated antibodies were added in fivefold serial dilution in FACS buffer and incubated at 4 °C for 45 min. Secondary antibody staining was done with goat antihuman IgE antibody conjugated with FITC (Novus Bio) in 1:1000 dilution in FACS buffer at 4 °C for 45 min. Samples were analyzed by FACS CantoTM II (BD) using the software program BD FACSDiva (BD Biosciences). EC50 values were calculated in GraphPad Prism (GraphPad Software).

To test the simultaneous antigen-binding activity of anti-PSMA x EGFR bsAb, the cell bridging experiment was used as described previously (19) with some modifications. To prepare a single-cell suspension, CHO/mPSMA and CHO/mEGFR cells were detached from flasks and washed twice with PBS. CHO/mPSMA and CHO/mEGFR cells were labeled with cell staining dye eFluor 450 or 670 (eBioscience), respectively, according to the manufacturer's recommendations. About 5×10^4 labeled CHO/mPSMA cells were mixed in 50 μ l FACS buffer and incubated with indicated concentrations of anti-PSMA x EGFR bsAb at 4 °C for 45 min. The cells were washed three times with FACS buffer and 5×10^4 labeled CHO/mEGFR cells were added. The cells were incubated at 4 °C for 45 min and analyzed on a FACS CantoTM II (BD) using the software program BD FACSDiva.

Human MC generation

Human peripheral blood mononuclear cell (PBMC)-derived MCs were generated from buffy coats as previously described by Folkerts *et al.* (34) Buffy coats were obtained from healthy donors (Sanquin). Before sample collection, written consent was obtained. In short, buffy coats were used to obtain PBMCs, after which CD34⁺ precursor cells were isolated using the EasySep Human CD34 Positive Selection Kit (STEMCELL Technologies). CD34⁺ cells were cultured for 4 weeks under serum-free conditions in StemSpan medium (STEMCELL Technologies) supplemented with recombinant human IL-6 (50 ng/ml; Peprotech), human IL-3 (10 ng/ml; Peprotech), and human Stem Cell Factor (100 ng/ml PeproTech). After

4 weeks, the cell culture medium was changed to Iscove's modified Dulbecco's medium supplemented with 0.5% bovine serum albumin, human IL-6 (50 ng/ml, PeproTech), and 3% supernatant of CHO transfectants secreting murine stem cell factor (a gift from Dr P. Dubreuil). The mature MCs were identified based on the expression of CD117 (eBioscience) and Fc ϵ R1a (eBioscience) by flow cytometry using BD FACS Canto II (approximately 90%).

Human MC degranulation assay (β -hex assay and IL8 ELISA)

CHO/mEGFR and CHO/mPSMA cells were plated in 96-well plates at the density of 10,000 and 15,000 cells/well, respectively, and cultured for 2 days to ~80% confluency. Human PBMC-derived MCs were primed with the indicated concentrations of IgE antibodies at 37 °C overnight, after which the unbound IgE was washed, and sensitized MCs were either added to plated CHO cells washed with PBS (20,000 MCs/well) or the crosslinking was induced with 1.25 to 10 μ g/ml antihuman IgE antibody (Dako; #A0094). Myeloma IgE clone AG30P (Merck Millipore) was used as a negative control. After 2 h, supernatants were collected and either used immediately in the β -hex assay or the aliquots were frozen for IL-8 quantification.

For β -hex assay, the supernatants were mixed with 200 μ M 4-methylumbelliferyl- β -d-glucosaminide substrate in 100 mM citric acid, pH 4.5, for 1 h at 37 °C. The enzymatic reaction was stopped with 0.1 M glycine buffer, pH 10.7. As a positive control, cells were lysed with 0.2% Triton X-100, in order to determine total β -hex content. The β -hex content was quantified by fluorometric measurements at ex360/em452 nm. The percentage of released β -hex was calculated by using the following equation: $\frac{A-B}{T-B} \times 100\%$, where A is the amount of β -hex released from stimulated cells, B is the amount of β -hex released from unstimulated cells, and T is total β -hex content.

IL-8 was quantified with the IL-8 ELISA kit (Invitrogen; #88-8086-88), as per the manufacturer's recommendations.

Human subjects and sample collection for ADCC

This study was approved by the Clinica Universidad de Navarra Ethics Committee (2019.139) and Navarra Blood and Tissue Bank Navarrabiomed Biobank. We have complied with all relevant ethical regulations for working with human participants in accordance with the Declaration of Helsinki.

Isolation of human CD11b⁺ cells from donor blood

To isolate PBMCs, the donor blood was submitted to Ficoll-Paque (Cytiva; 17144003) gradient centrifugation, after which the buffy coat was collected, washed with PBS, and submitted to red blood cell lysis with ammonium-chloride-potassium lysing buffer (Gibco; A1049201). After stopping the lysis reaction, the PBMCs were passed through a 70 μ m cell strainer and washed with PBS. Next, PBMCs were used to isolate CD11b⁺ cells using CD11b MicroBeads (Miltenyi Biotec; 130-049-601) as per the manufacturer's protocol.

ADCC assay

The ADCC assay was performed in xCELLigence Real-Time Cell Analysis Instrument (Agilent, xCELLigence RTCA DP), an impedance-based technology that monitors cell proliferation and attachment in real-time. The day before starting the assay, target CHO/mPSMA_mEGFR cells were plated in E-Plate 16 PET (Agilent #300600890), 40,000 cells/well, to allow them to adhere and reach the exponential growth phase. The following day the human CD11b⁺ effector cells were pre-incubated with indicated IgE/IgG antibodies at 5 µg/ml for 1 h, at 37 °C and 5% CO₂, after which the unbound antibodies were washed, and 80,000 of IgE- or IgG-coated effector cells were added per well. The cell killing was monitored by the instrument.

In vivo cytotoxicity assay

BALB/c.Rag2^{tm1Fwa} IL2rg^{tm1Wjl} (Rag2^{-/-}γc^{-/-}) mice were bred and maintained in the animal facility of Centro de investigación médica aplicada (CIMA), Universidad de Navarra maintained at CIMA under the guidelines of the Ethics Committee of the center. CHO/mEGFR_mPSMA cells were detached and prestained with CellTracker Green CMFDA Dye (ThermoFisher Scientific; #C7025, 1:1500 dilution) according to manufacturer's protocol for cells in suspension. The mice were intraperitoneally injected with 1.5 × 10⁶ CMFDA-prestained CHO/mEGFR_mPSMA cells into the left side of the peritoneal cavity. Straight after, they received a separate injection into the right side of the peritoneal cavity containing 3 × 10⁶ human CD11b⁺ cells isolated from a fresh buffy coat as described previously, together with 50 µg of indicated bispecific antibodies. After 24 h, we performed the peritoneal lavage with 3 ml of PBS, and the cell populations were quantified by flow cytometry on CytoFLEX S (BD; B75408). The staining included antimouse CD45.2 (Biolegend #109820, 1:200) in order to exclude murine leukocytes, antihuman CD11b PerCP/Cy5.5 (Biolegend #301328, 1:100) for effector population detection, and Zombie NIR (Biolegend #423105, 1:1000) for live/dead. The gating strategy is shown in Fig. S2.

Statistical analysis

GraphPad Prism V.8.2.1 software (GraphPad Software) was used for statistical analysis. Mean differences were compared using *t* tests (for comparisons of two groups) or two-way ANOVA tests (for comparisons of three or more groups). Longitudinal data were fitted to a fourth-order polynomial equation and compared with an extra sum-of-squares F test. Values of *p* < 0.05 were considered to be statistically significant.

Data availability

All data generated in this study are available within the article and its supplementary data files.

Supporting information—This article contains supporting information.

Author contributions—N. V., S. H., and D. M. W. Z. conceptualization; N. V., S. H., P. B., F. A. M. R., and D. M. W. Z. methodology; N. V. formal analysis; N. V., J. S. R.-C., and B. B. investigation; P. B., F. A. M. R., and D. M. W. Z. writing—review & editing; N. V. visualization; D. M. W. Z. supervision.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: β-hex, β-hexosaminidase; ADCC, antibody-dependent cell cytotoxicity; bsAb, bispecific antibody; CHO, Chinese hamster ovary; CMFDA, 5-chloromethylfluorescein diacetate; EGFR, epidermal growth factor receptor; FACS, flow cytometry staining; FBS, fetal bovine serum; KiH, knobs-into-holes; LZ, leucine zipper; MC, mast cell; PBMC, peripheral blood mononuclear cell; PSMA, prostate-specific membrane antigen; sdAb, single domain antibody; TAA, tumor-associated antigen.

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Supplementary

Supplementary Table 1. Amino acid sequences of designed antibodies.

SAM103(anti-mPSMA) fos IgE heavy chain -> leucine zippers bsAb
MAVLGLLFLCLVTFPSCVLSEVKLVESEGGLVQPGSSMKLSCTASGFTFSDYYMAWVRQVPEKGLEWVANINYD GTTTTYYLDSLKSRFIISRDN SKN ILYLQMSSLKSEDTATYYCARVLDGYYGYFDYWGQGTTL SVSSASTQSPS VFPLTRCCKNIPSNATSVTLGCLATGYFPEPVMVTWDTGSLNGTTMTLPATTLTSLSGHYATISLLTVSGAWAK QMFTCRVAHTPSSTDWVDNKTFSVCSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDG QVMDVDLSTASTTQEGELASTQSELTLSQKHWLSDRYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRPS FDLFIRKSPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTLPVGTRDWIEGETYQC RVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLACLIQNFMPEDISVQWLHNEVQLPDARHS TTQPRKTKGSGFFVFSRLEVTRAWEQKDEFICRAVHEAASPSQTVQRAVSVNPGKGSAWSHPQFEKGGGLVP RSGSGGSLD TDLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAA
SAM103(anti-mPSMA) S10I IgE heavy chain -> KiH bsAb
MAVLGLLFLCLVTFPSCVLSEVKLVESEGGLVQPGSSMKLSCTASGFTFSDYYMAWVRQVPEKGLEWVANINYD GTTTTYYLDSLKSRFIISRDN SKN ILYLQMSSLKSEDTATYYCARVLDGYYGYFDYWGQGTTL SVSSASTQSPS VFPLTRCCKNIPSNATSVTLGCLATGYFPEPVMVTWDTGSLNGTTMTLPATTLTSLSGHYATISLLTVSGAWAK QMFTCRVAHTPSSTDWVDNKTFSVCSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDG QVMDVDLSTASTTQEGELASTQSELTLSQKHWLSDRYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRPS FDLFIRKSPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTLPVGTRDWIEGETYQC RVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLACLIQNFMPEDISVQWLHNEVQLPDARHS TTQPRKTKGSGFFVFSRLEVTRAWEQKDEFICRAVHEAASPSQTVQRAVSVNPGKGSAWSHPQFEK
SAM103(anti-mPSMA) IgE heavy chain -> monospecific parental Ab
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SAM103(anti-mPSMA) kappa light chain
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RR359(anti-mEGFR) jun IgE heavy chain -> leucine zippers bsAb
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RR359(anti-mEGFR) T121G IgE heavy chain ->KiH bsAb
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RR359(anti-mEGFR) IgE heavy chain -> monospecific parental Ab
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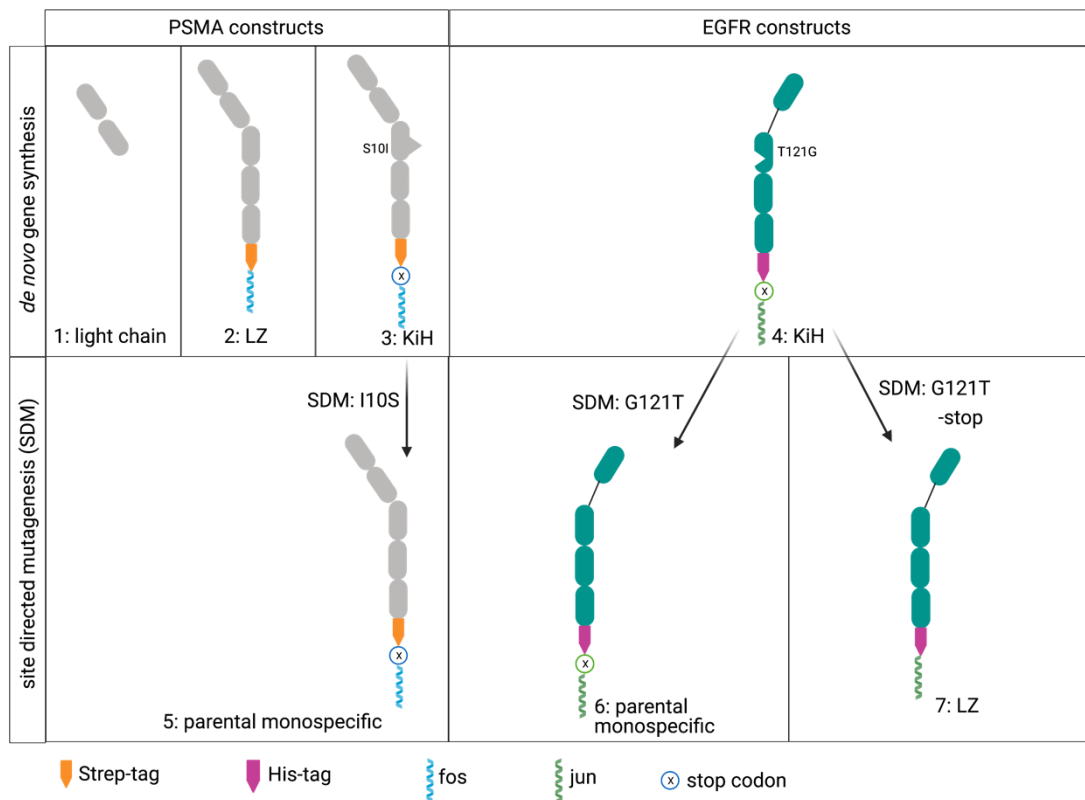
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SAM103(anti-mPSMA) T366W KiH IgG1 heavy chain

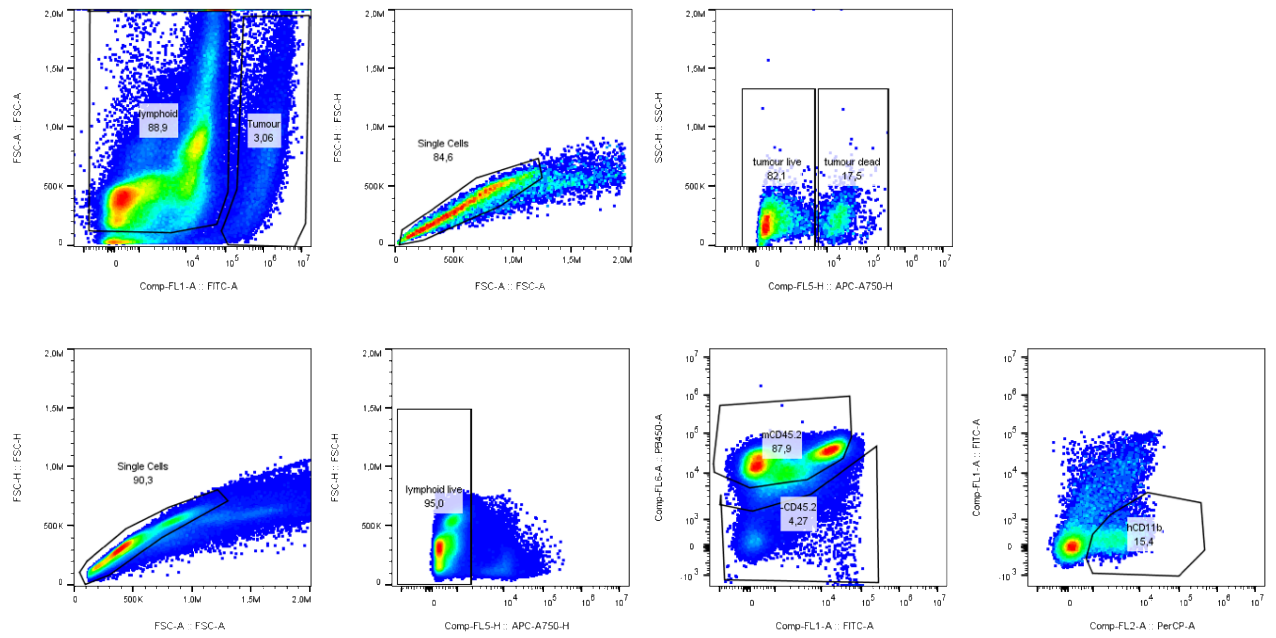
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RR359(anti-mEGFR) T366S, L368A and Y407V KiH IgG1 heavy chain

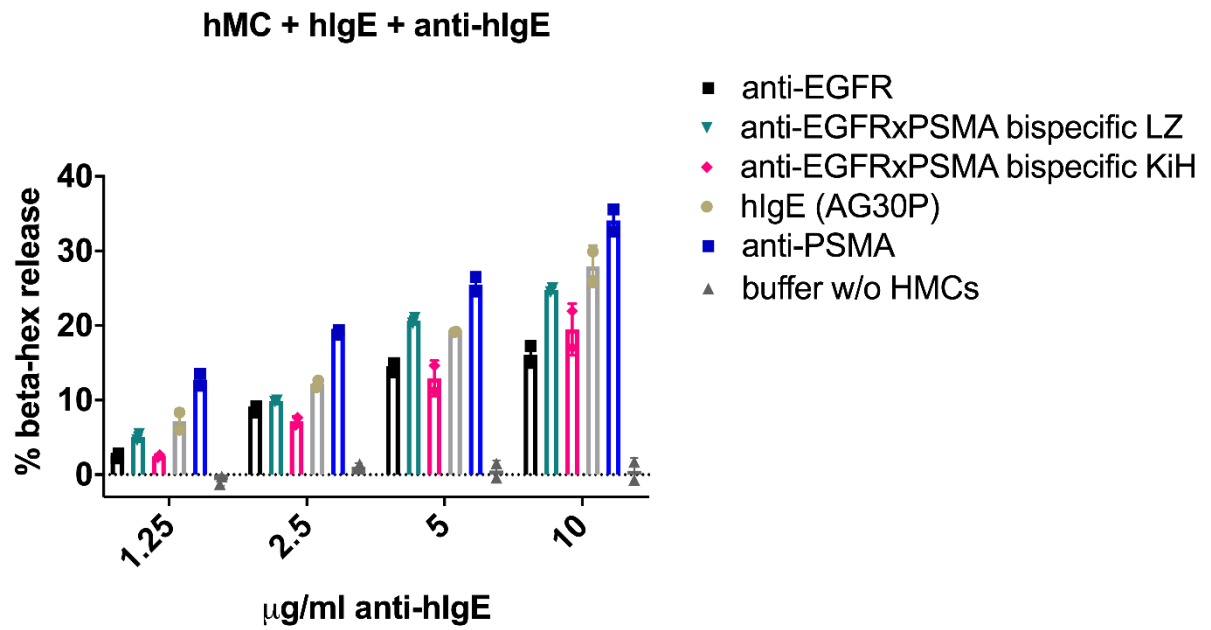
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 VSSEPKTKPKPQPQPQPKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFN
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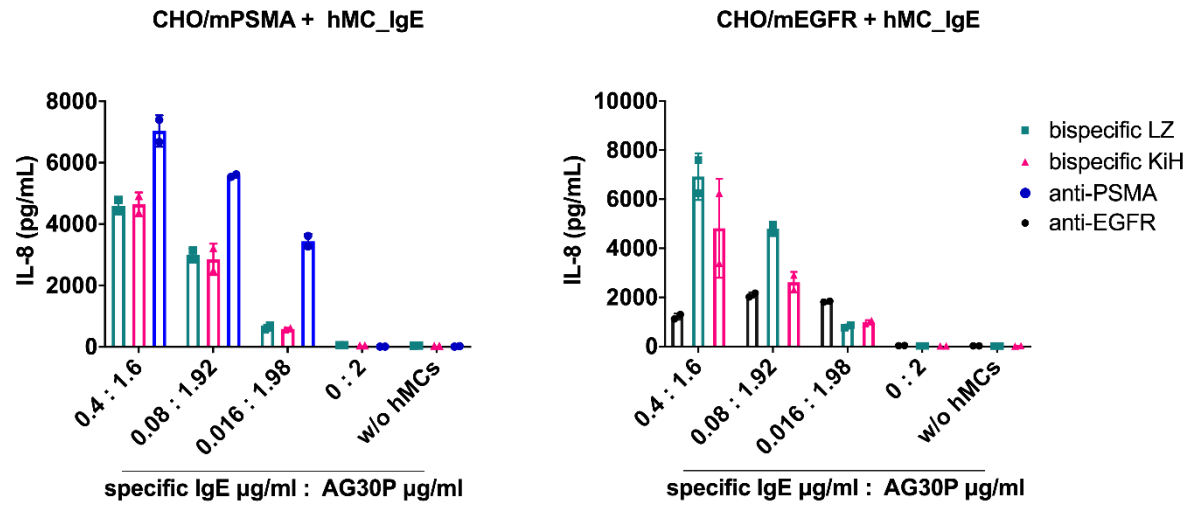
Supplementary Figure 1. Antibody DNA constructs were obtained by either *de novo* gene synthesis or site directed mutagenesis.



Supplementary Figure 2. Gating strategy for flow cytometry analysis of peritoneal lavages.

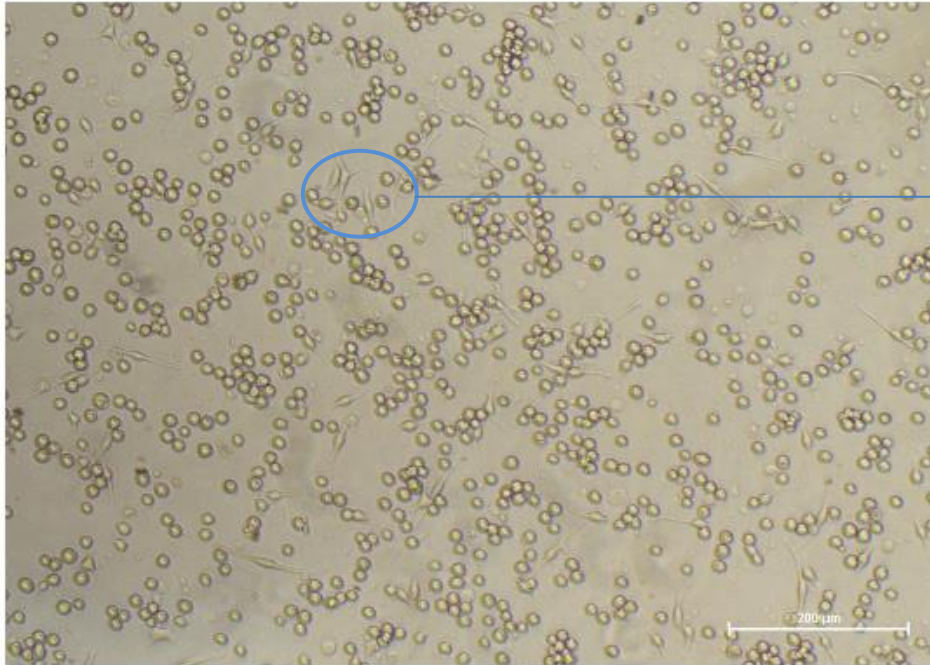


Supplementary Figure 3. Bispecific IgEs induce mast cell degranulation. Human mast cells were sensitized with indicated antibodies and the cross-linking was induced with anti-human IgE antibody in a dose-dependent manner.



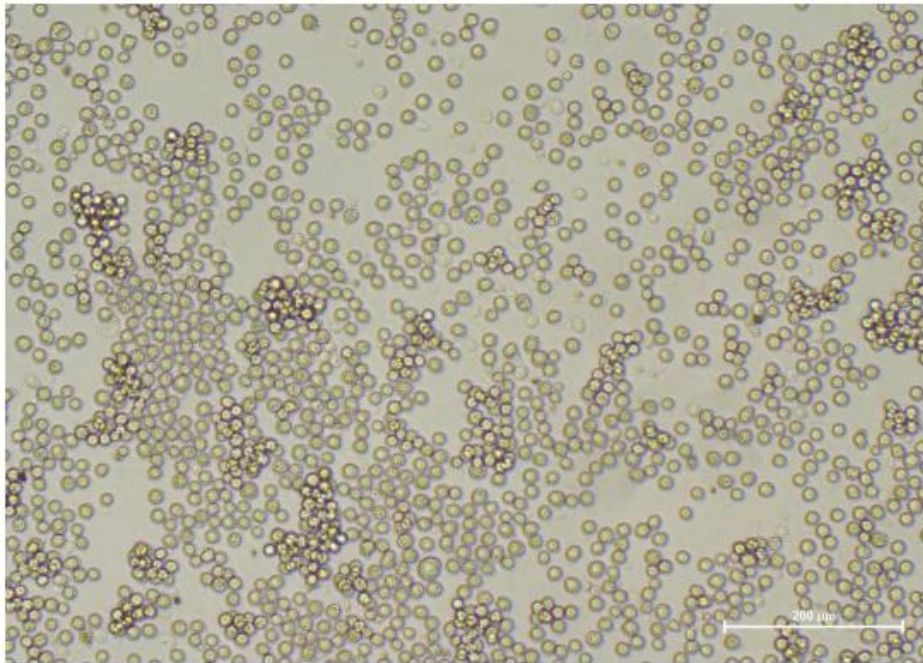
Supplementary Figure 4. IL-8 levels measured in the supernatant after MC degranulation

HMC_EGFR_IgE



Activated MCs

HMC_PSMA_IgE



Supplementary figure 5. MC morphology after sensitization step with indicated IgE antibodies. Scale bars correspond to 200μm.