UNIVERSITA' DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO IN SCIENZE FARMACOLOGICHE

Corso di Dottorato in Farmacologia, Chemioterapia e Tossicologia Medica

Settore Scientifico Disciplinare BIO/14

Ciclo XXV

Generation of a transgenic FcεRI-KO/ high IgE-producer mouse to elucidate the role of IgE in tumor surveillance

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Anno Accademico 2012/2013

ABSTRACT

Immunoglobulin E (IgE) is a powerful elicitor of anti-parasitic immunity and play a key role in pathophysiology of allergic reactions.IgE antibodies acts by engaging both high and low affinity receptors, FcεRI and CD23. Since the 1960s a number of epidemiological studies have investigated the potential association between a history of allergy and risk of malignancy. In this direction studies were planned by redirecting the power of the IgE system towards tumors, and to evaluate if it can trigger powerful anti-tumor cytotoxic activities. Previously in our laboratory,mouse and human IgE have been used as vaccines adjuvants,highlighting the importance of IgE:FcεRI interaction in establishment of strong allergy-like inflammatory response at tumor site,with induction of adaptive antitumor immune response and memory.

Based on these results and Epidemiological studies relating possible link between allergies and cancer protection, we decided to study the involvement of endogenousIgE with antitumor specific IgE effect *in vivo* making use of high IgE producing(KN1-HyperIgE) and IgE *knock out* transgenics and two different tumor cell lines (TS/A-LACK and N2C), upon challenge we were able to note that tumor growth was seen in IgE *knock out* mice, *wild type* control mice and CD-23 *knock out* mice, however there was a striking tumor protection effect in KN1-HyperIgE mice. The results from *in vitro* studies showed presence of tumor specific IgE antibodies in serum of only KN1-HyperIgE (high IgE producing) group indicating the role of IgE mediated mechanism in antitumor protection. In order to prove the involvement of Endogenous IgE in antitumor protection observed *in* vivo, we investigated if Bone Marrow cells of KN1-HyperIgE producing mice when transplanted can induct protection in spontaneously tumor developing mice(HER-2/neuT),BMT experiments were performed using the bone marrow cells from KN1-HyperIgE mice and failed to have delayed and/or desired antitumor effect in immunocompromised spontaneous tumor developing HER-2/neuT mice. To validate that indeed IgE is involved in tumor protection and this protection is mediated by its interaction with its high affinity receptor FcεRI, a new transgenic was generated by breeding KN1-HyperIgE and FcεRIα *knock out* mice to arrive at a Double mutant(DB) mice(phenotype being high IgE producing but lacking in functional high affinity receptor FcεRI). In experiment with tumor challenge, there was tumor protection in HyperIgE mice, but was lost in the double mutant mice inducting the role of high affinity receptor FcεRI in tumor protection. Even the in vitro results showed the release of antitumor specific IgE antibodies in KN1-HyperIgE(high IgE producing) as well as Double mutant group indicating that tumor protection lost in Double

mutant mice is due to absence of functional high affinity receptor FcεRI .In a ongoing experiment, depletion of CD8+ cells led to abolishment of tumor protection in KN1-HyperIgE mice.

The results from this thesis study clearly signify the role of IgE in antitumor mechanism and highlight the prominent role of IgE:FcεRI in induction of tumor protection. It also points towards crucial role of CD8+cells in antitumor immunity, suggesting towards an IgE mediated cross presentation pathway for induction of TAA specific cytotoxic lymphocytes in antitumor response. Studies are ongoing to further elucidate the evolving role IgE mediated mechanism in the context of cancer.

Introduction

Cancer is a group of diseases and is an outcome of uncontrolled growth of abnormal cells with subsequent invasion into surrounding tissues, leading to metastasis. Cancer malignancies are life-threatening and one of the leading causes of death. Annually about 1% of population diagnosed with cancer, die. According to information available from National Cancer Institute,USA the survival rates for five years is 60-80% for breast and prostate cancer,40-60% for bladder, colon and cervix cancer and 10-20% in case of stomach.eoshagus and lung cancer (www.rex.nci.nih.gov).

Mainly people diagnosed with Cancer undergo combination of surgery, radiation and/or chemotherapy. In most cases the primary tumor has been efficiently treated by these practices but the spread of metastasis by means of few disseminated tumor cells has not been effective. Therefore eradication of these breakaway tumor cells present in blood circulation and of small metastases in distant organs is the objective of Cancer Immunotherapy [Schuster *et al*., 2006].

Cancer immunotherapy primarily aims to prevent the metastatic spread of the disease. It exploits the therapeutic potential of tumor-specific antibodies and cellular immune effector mechanisms (Schuster *et al*., 2006:Yan L,2006) Approaches are based on complementation or stimulation of the immune system by using vaccines, antibodies or lymphokines (Mocellin *et al*.,2007). Immune surveillance is the concept that aims in preventing the tumor development by destroying the abnormal cells through the immune system of the host (Swann *et al*.,2007). In Passive Immunotherapy, repeated tumor-specific antibodies are administered for immune response, whereas Active immunotherapy stimulates host's immune response following vaccination (Katsumata *et al*.,1995; Nanni *et al*., 2001) It is thus with hope that new vaccines based on innovative technologies will progressively reach the efficacy of most conventional cancer therapies and help patients from the devastating side effects of chemotherapy. In some cases, the cure afforded by a vaccine may prove to be even more effective and persistent than chemotherapy (Schuster *et al.,* 2006).

Recent evidences also point that immunotherapy is becoming a good option in management of cancer patients. The results show they are equal or better than most successful conventional treatments in range of neoplastic diseases such as lymphoma(Schuster *et al.,*2009,melanomas(Schwartzentruber *et.al*.,2009,prostate cancer(Couzin-Frankel, 2010)

Many of current preclinical and clinical results converge on the relevance of antibodies in antitumor immune responses (Lollini *et al.,* 2011; Nelson *et al.,* 2010; Weiner *et al.,*2010).

1.1 Antibodies in treating of Cancer

Paul Ehrlich in 1900's was the first to use antibodies as ''magic bullet'' to specifically target malignant cells. The inherent specificity of antibodies would result in enhanced antitumor activity while reducing the non-specific side effects that are inevitably associated with conventional chemo and radiotherapy. Antibodies offer some advantages over small molecule drugs for the treatment of cancer and other diseases, for their potential for low toxicity, improved pharmacokinetic profiles and specificity (Ang *et al.*,2012).

The concept that the injection of therapeutic antibodies not only triggers early anti-tumor events such as receptor blockade, cytostasis, apoptosis, complement dependent cytotoxicity and/or antibody-dependent cytotoxicity but also allows the host immune system to fight tumor cells through the development of a long-lasting adaptive immunity has emerged. Immunosurveillance is a critical mechanism in the control of tumor development. Notably, the presence of CD8+ effector/ memory T cells was positively correlated with a favorable prognosis in a large number of cancer patients([Abès *et al.*,2011).

However, antibodies as cancer therapeutics can also have limitations such as poor tumor penetration, poor oral bioavailability, and high cost associated with manufacture. This problem has been successfully solved by generation of Monoclonal antibodies (mAbs). They have emerged as an important drug class in pharmaceutical development and clinical practice. The principle advantages of mAb over the conventional polyclonal antibodies are in their defined specificity, homogeneity, and availability of the mAb in practically unlimited quantities. These properties of mAb render them as one of the most attractive classes of therapeutic agents in treatment of malignant tumors.

Since 1986, the FDA has approved 20 therapeutic mAb for clinical use in the United States, including 12 for oncology currently being used.mAbs also functions as potent and specific molecular antagonists. These mAbs are being developed as cancer therapeutics to block molecular functions, elicit immune effector functions, or as immunoconjugates for tumor-specific drug delivery (Yan,2006).

1.2 The Structure of Antibodies

Antibodies are immune system-related proteins called immunoglobulins. Immunoglobulins are glycoprotein molecules that are produced by plasma cells in response to an immunogen.

Antibodies acts as antigen receptors (B cell receptor or BCR), bound to the surface membrane of B cells, or they may be secreted as soluble proteins and bind to specific antigen. Each Antibody actually binds to a specific antigenic determinant. Antigen binding by antibodies is the primary function of antibodies and can result in protection of the host. The binding of an antibody to an antigen has no direct biological effect, rather the significant biological effects are a consequence of “effector functions" of antibodies (Jiang *et al.*,2006)

Basic structure of the Immunoglobulins (Ig’s)

Each antibody consists of four polypeptides– two heavy chains and two light chains linked by disulfide bonds and non-covalent interactions to form a "Y" shaped molecule (Woof *et al.,*2004)

1. **Heavy and Light Chains**

All immunoglobulins have a four chain structure as their basic unit. They are composed of two identical light chains (23kD) and two identical heavy chains (50-70kD)(Ferrara *et al.*,2004)

There are 5 different classes of antibody and these classes are defined by the class of **Heavy Chain**

IgM -- μ chain – 5 domains

IgG -- γ chain – 4 domains

IgA -- α chain – 4 domains

IgE -- ε chain – 5 domains

IgD -- ð chain – 4 domains

Ig heavy chains are comprised of one variable domain (VH) and multiple constant domains (CH), three for IgA, IgG and IgD and four for IgE and IgM.

There are 2 classes of **Light Chains**

λ chain – 2 domains – lambda

k chain – 2 domains – kappa

For light chains only one type, κ or λ, is present per antibody. Light chains are also composed of one variable domain (VL); however, in contrast to the heavy chains, they only have a single constant domain for all antibody classes

2. Disulfide bonds

a. Inter-chain disulfide bonds - The heavy and light chains and the two heavy chains are held together by inter-chain disulfide bonds and by non-covalent interactions. The number of inter-chain disulfide bonds varies among different immunoglobulin molecules.

b. Intra-chain disulfide binds - Within each of the polypeptide chain there are also intra-chain disulfide bonds.

3. Variable (V) and Constant (C) Regions

When the amino acid sequences of many different heavy chains and light chains are analysed, it is clear that both the heavy and light chain could be divided into two regions based on variability in the amino acid sequences(Woof et al.,2004). These are the:

a. Light Chain - V_L (110 amino acids) and C_L (110 amino acids)

b. Heavy Chain - V_H (110 amino acids) and C_H (330-440 amino acids)

4. Hinge Region

This is the region at which the arms of the antibody molecule form a Y. It is called the hinge region because there is some flexibility in the molecule at this point.

5. Domains

3D images of the immunoglobulin molecule show that it is not straight and is folded into globular regions each of which contains an intra-chain disulfide bond. These regions are called domains.

a. Light Chain Domains - V_L and C_L

b. Heavy Chain Domains - V_H, C_{H1} - C_{H3} (or C_{H4})

6. Oligosaccharides

Carbohydrates are attached to the C_{H2} domain in most immunoglobulins. However, in some cases carbohydrates may also be attached at other locations.

Although antibody structure is similar among the five Ig classes, there are key differences among them that can be grouped into 3 main categories:

a. the number of heavy chain domains

b. the distribution and number of carbohydrate groups, and

c. the number and location of disulfide bonds linking the different domains.

The variable domains show three regions of hyper variability in sequence called the complementarity determining regions (CDRs). They differ in length and sequence between different antibodies and are mainly responsible for the specificity (recognition) and affinity (binding) of the antibodies to their target markers. Antibodies can be divided into two distinct functional binding units. Proteolytic digestion of antibodies releases different fragments termed Fab (Fragment antigen binding) and Fc (Fragment crystallization)

Fab-Region of Antigen binding - These fragments are called the Fab fragments because they contain the antigen binding sites of the antibody. The combining site of the antibody is created by both V_H and V_L. An antibody is able to bind a particular antigenic determinant because it has a particular combination of V_H and V_L. Different combinations of V_H and V_L result in antibodies that can bind different antigenic determinants.

Fc-This fragment is called Fc because it is easily crystallized. The effector functions of immunoglobulins are mediated by this part of the molecule

1.3 Mechanism of action of Antibodies in cancer therapy

The study reported by Galon and his colleagues with tumors derived from hundreds of patients has shown that the development of colon carcinoma in human's results from a complex process where tumors cells and immune cells are engaged in a mortal combat, demonstrating that immuno-surveillance is a critical mechanism in the control of tumor development. The surveillance of tumors by the immune system of cancer patients and its impact on disease progression and patient survival has been largely documented (Abès *et al.,*2004). The role of the antibodies include

- Modulating the function of key regulatory molecules on tumor cells such as blocking growth factor/ receptor interaction and/or down-regulating expression of oncogenic proteins (e.g., growth factor receptors) on the cell surface (Ferrara *et al.,* 2004).
- Recruiting effector mechanisms of the immune system, such as antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC) and antibody dependent cellular phagocytosis (ADCP) (Lewis *et al.,*1993; Reff *et al.*,1994).
- As a targeting device in immunoconjugates, the antibody acts as a carrier molecule to deliver an attached chemotherapeutic agent, toxin, or radio-isotope to cells displaying a specific antigen (Siever *et al.*,2001).

- Other mechanisms, e.g., antibodies that stimulate the anti-idiotype network to generate anti-tumor anti-anti-idiotypic antibody response (Bhattacharya-Chatterjee *et al*., 2002).

Monoclonal antibodies have emerged as novel oncology therapeutics. These biologics exert anticancer effects via a variety of mechanisms (Yan L., 2006). Also now that the ability to produce therapeutic antibodies that are compatible with the human immune system has improved, mAbs have begun to play a larger role in the treatment of oncologic disorders. Antibody engineering has allowed investigators to improve tumor penetration through the construction of smaller, multivalent antibody-based molecules (Spillner *et al*., 2012; Chan *et al*., 2012; Chames *et al*.,2009). Now they try to target mAb to solid tumors with sufficient specificity and in high enough concentration to make them effective therapeutics (Robinson *et al*.,2004) mAbs, like rituximab for the treatment of Non-Hodgkin's lymphoma, are approved for the treatment of blood-borne cancers (Daniels *et al.,* 2012; Garrido *et al.,* 2007). Trastuzumab, is approved for the treatment of metastatic breast cancer and is the only antibody-based therapy approved for the treatment of solid tumors[Daniels *et al*.,2012]. By creating chimeric (variable regions transferred onto human Fc region) or humanized (complimentarity- determining regions, CDRs, grafted onto a human antibody, Almost all of the mAbs currently approved by the U.S. Food and Drug Administration (FDA) are either chimeric or humanized mAbs (Robinson *et al*.,2004).

mAbs were originally developed to confer passive immunity against tumor cells via targeting tumor associated antigens. Now it has been recognized that mAbs can also function as potent and specific molecular antagonists (Yan l.,2006). Currently, mAbs are being developed as cancer therapeutics to block molecular function, elicit immune effector function, or as immunoconjugates for tumor-specific drug delivery. The effectiveness of an antibody depends on its capability to induce one or more of several biological mechanisms (Carter ,2001).

It is now well established that many anti-tumor mAbs require the recruitment of cells from the innate immune system to achieve therapeutic efficacy in vivo (Abès *et al.,*2011). Among them, FcγR expressing cells play a dual role since they include both potent cytotoxic cells (NK cells, macrophages, neutrophils) as well as dendritic cells (DC's) that allow the recruitment of specific anti-tumor T cells, possibly leading to the generation of an adaptive immune response(Abès *et al*., 2010). A number of strategies are being explored to further optimize the therapeutic efficacy of mAb. For example, given the importance of ADCC in the anti- tumor activity of a therapeutic antibody, manipulating the interaction between the Fc region and its receptor, FcγR, represents an

excellent approach to increase its tumor-killing activity .Molecular engineering of the Fc domain has been utilized to improve the affinity of a mAb for FcγRIII (Lazar *et al*., 2006; Shields *et al.,* 2001). Alternately, modifications of the glycosylation of the Fc domain by either addition of bisecting N-acetylglucosamine or deletion of fucose on N-linked oligosaccharide chain have al so been shown to enhance the binding affinity between the Fc domain and its FcγR(Shinkawa *et al*.,2003; Umana *et al*., 1999). Increased affinity between the Fc domain and the FcγR, especially FcγRIII, usually leads to enhanced ADCC activity of the engineered mAb (Lazar *et al*.,2006) Also cytokines such as IL-2, IL-12, and granulocyte macrophage colony-stimulating factor are known to enhance ADCC by stimulation of NK cells and macrophages (Cartron *et al.,* 2004).

Engagement of the adaptive immune system against tumor antigens represents a long-standing goal in cancer therapy. The concept that therapeutic mAbs not only trigger early anti-tumor events such as direct effects on tumor cells, or innate immune-mediated cell killing mechanisms, but that they also allow the host immune system to mount an anti-tumor response through the development of a long-lasting adaptive immunity has emerged (Kipriyanov *et al*., 2004; Kontermann *et al.,* 2005).

1.4 Antibodies in Therauptics

Although there are five antibody classes in humans, each with distinctive functions in the immune system, essentially all monoclonal antibodies approved for clinical use are IgG1's due to their favorable pharmacokinetic properties and pioneering experiments by Neuberger and colleagues, furthermore they have demonstrated that IgG, and in particular IgG1(Scott *et al.*,2012), was more effective than other antibody isotypes in activating complement and promoting ADCC and CDC against tumour cells *in vitro* (Bruggemann *et al.*,1987; Rodeck *et al* .,1987) Different IgG antibody subclasses however, have been employed therapeutically, such as IgG1, IgG2 and IgG4 depending on the desired role of the Fc-mediated mechanisms of action. For example, the IgG1 Fc region generally has the highest affinity of all of the IgG antibody subclasses for Fc receptors (FcγRs) on immune effector cells, leading to more potent effector functions (Bruggemann *et al.*,1987). On the other hand, IgG4 has weaker affinity for FcγRs, which translates into weaker or ineffective effector functions. Therefore IgG4 mAbs are used therapeutically when Fc-mediated effector functions are not desirable (Jiang, X *et al.*,2011). Furthermore, there has been some exploration of the use of mAbs of alternate Ig classes such as IgA and IgE in cancer

immunotherapy, with the aim of enhancing Fc-mediated effector functions by the engagement of alternative FcRs on immune effector cells such as FcαRI, FcεRI, and CD23.

Antibodies of the IgG class function most effectively in the circulation (Riethmuller G *et al.*,1992). There are many reasons why IgE antibodies might be more effective against tumors that develop in tissues and are therefore inaccessible to IgGs (Karagiannis *et al*., 2011). The concentration of IgE in the serum of normal individuals is minute (<150 ng/mL, 1/ 10,000 the concentration of IgG (5-10mg/ml)) because IgE is sequestered in solid tissues, where it is bound with high affinity to receptors on its effector and antigen-presenting cells [Waldmann *et al*., 1975].This low concentration is because of IgE's half life (1-3 days as compared to 3 weeks of IgG) and small number of IgE-secreting plasma cells(lanzavecchia et.al).Furthermore, circulating IgE is captured by B cells via CD23 receptor and becomes tissue resident IgE with half-life of 1-2 weeks (Gould *et.al.,* 2003)

The affinity of IgE for FcεRI (Ka =$10^{11}M^{-1}$) is 10^{2}–10^{5} times higher than those of IgGs for their receptors, making IgE the only antibody strongly retained by effector cells in the absence of antigen(Waldmann et al., 1975). IgE antibodies on the surface of tissue mast cells are cross-linked by antigens to induce the release of histamines, leukotrienes, proteases, and, importantly, Th2 cell-type cytokines (IL-3, IL-4, IL-5, IL-6, IL-9, IL-13, TNF-α) at the site of antigen challenge. This results in activation of the resident immune effector cells, but also elicits further recruitment and persistence of an inflammatory cell infiltrate, comprising Th2 cells, monocytes, eosinophils and basophils, from the circulation, which enhances and maintains the local immune response]. IgE antibodies directed against a tumour associated antigen would therefore trigger an immediate local effector cell response against tumour cells and stimulate a cascade of inflammation targeted to the tumour cells in situ. These activities could possibly be highly effective in immune rejection of tumours embedded in solid tissues. Several studies support the idea that IgE antibodies may be highly effective tools in cancer therapy (Riemer *et al.,*2007; Jensen-Jarolim E *et al*., 2008; Reali E *et al.,*2001; Turner *et al* 2005-2006;).

1.5 IgE in Immune Responses

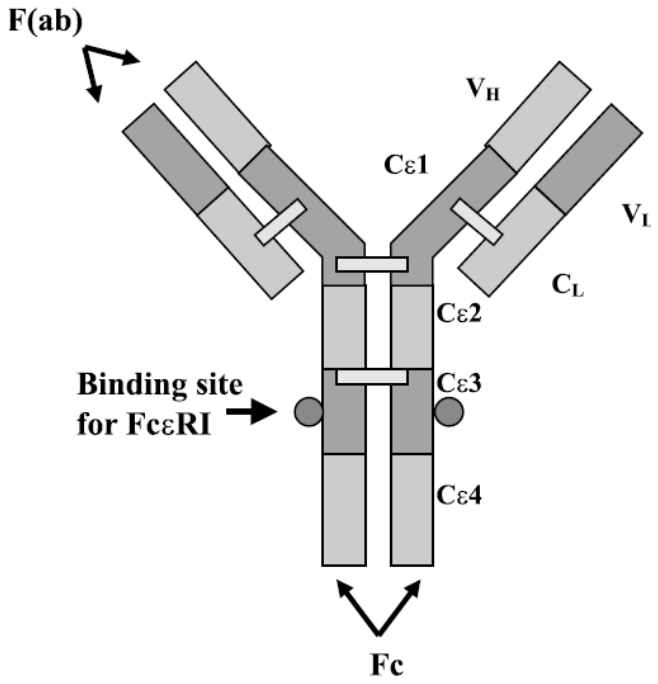


Figure 1 The structure of IgE. IgE molecules are composed of two identical heavy and light chain pairs, which are linked by disulfide bonds. The heavy chains consist of four constant domains (Cε1, Cε2, Cε3 and Cε4 and one variable domain (VH). Light chains also contain one variable domain (VL) and one constant domain (CL). Antigen binding sites are comprised of the VH and VL domains of the Fab region,.Effector function is mediated by binding of the Fc region in the heavy chains to complement or to cellular Fc receptors. Figure adapted from (Hammelmann,2002).

IgE was the last of the immunoglobulins discovered. IgE is an evolutionary conserved member of the Ig family with the highest determined affinity to receptors and antigens among all antibody classes(Gould *et al.*, 2003). IgE is most prominent in epithelia and mucosa where it is bound to receptors on very potent effector cells like eosinophil or basophil granulocytes and mast cells (Keegan *et al.*,1990; Macglashan *et al.,* 2005).

IgE is known to play a role in allergic inflammatory processes such as anaphylaxis, allergic rhinitis, asthma and atopic dermatitis(Zhang M *et al.*, 2007).IgE is produced by plasma cells located in lymph nodes, draining the site of antigen entry or locally, at the sites of

allergic reactions, by plasma cells derived from germinal centers developing within the inflamed tissue.

The IgE molecule shares the same basic molecular architecture of antibodies of other classes, with two identical heavy chains and two identical light chains; the heavy ε-chain contains one more domain than the heavy γ-chain of IgG. The pair of Cε3 and Cε4 domains is homologous in sequence, and similar in quaternary structure, to the pair of Cγ2 and Cγ3 domains of IgG, so that it is the pair of Cε2 domains, located in the position equivalent to that occupied by the flexible hinge region of IgG that is the most obvious distinguishing feature of IgE. Each heavy chain of all membrane IgE molecules contains an additional extracellular membrane-proximal domain (EMPD) between the Cε4 domain and the transmembrane sequence.

IgE network includes its two principal receptors, **FcεRI** -high-affinity Fc receptor for IgE and **CD23**- low-affinity IgE receptor ***(FcεRII)***.

FcεRI

The high-affinity IgE receptor (FcεRI) is expressed on mast cells and basophils as tetramers (αβγ2) and on antigen-presenting cells, at lower levels, as trimers (αγ2).

Tetrameric FcεRI is composed of an α-chain, a β -chain and a disulfide linked γ-chain homodimer (αβγ2), whereas for trimeric expression, the α-chain associates with the γ2-dimer in the absence of a β-chain (αγ2).Tetrameric and trimeric FcεRI isoforms have different cellular expression patterns associated with different IgE effector functions. The αβγ2 tetramer is expressed only on basophils and MCs, where it is expressed in abundance (~ 200,000 molecules/cell), whereas the αγ2 trimer is expressed on Langerhans cells, myeloid DCs, plasmacytoid DCs, monocytes and eosinophils, at 10 – 100 fold lower concentrations (Macglashan *et al.,* 2005). Expression of the β chain in mast cells and basophils results in increased FcεRI surface expression and amplifies signaling through the receptor. In mouse, the expression of FcεRI is restricted to mast cells and basophils in its tetrameric structure.(Kinet *et al.,*1999)

The two extracellular immunoglobulin domains of the α chain contain the IgE binding region (the membrane-proximal domain) and the molecular information for an efficient folding (the N-terminal-domain), whereas the signaling motifs are located in the intracellular sequences of the β and γ chains(Gould.*et.al* 2008). The FcεRI subunits have no known enzymatic activity but rather signal through associated cytoplasmic tyrosine kinases. The chain of FcεRI binds to the Fc portion (C3 domain) of IgE and consists of an extracellular domain, a transmembrane

domain, and a short cytoplasmic tail with no signaling motifs. The β subunit consists of 4 transmembrane domains with a single immunoreceptor tyrosine–based activation motif (ITAM) and is associated with Lyn kinase. The γ subunits form a disulfide-linked dimer, and each subunit contains an ITAM. After aggregation of FcεRI by multivalent antigen recognized by bound IgE, Lyn phosphorylates tyrosine residues in the ITAMs of the β and γ subunits. The tyrosine-phosphorylated γ subunit then recruits Syk kinase. Syk activates a number of downstream signaling events associated with mast cell or basophil activation (Turner *et al*., 1999; Taylor *et al* ., 1995) Syk is the target for a number of experimental therapeutic agents.

Binding of polyvalent antigens by receptor-bound IgE causes receptor aggregation and triggers cellular activation. This leads to the release of inflammatory mediators and vasodilatators from effector cells of the allergic reaction. Activation of eosinophils by FcεRI provides defense mechanisms against parasitic infection, whereas FcεRI on antigen-presenting cells can deliver IgE-bound antigen into MHC class II antigen-presentation pathways(Kraft *et al.,* 2007).

CD23/ FcεRII

The low-affinity IgE receptor (FcεRII named also CD23) is expressed on the surface of B cells, as well as other hematopoietic cells. It is a type II integral membrane protein, which belongs to the calcium-dependent (C-type) lectin superfamily. Its expression can be induced on a broad range of immune cells such as activated B cells, activated monocytes and macrophages, eosinophils, natural killer T cells, T cells, follicular DCs and platelets, but also on non-immune cells, such as airway epithelial cells and smooth muscle cells (Gould *et al.,* 2008; Dullaers *et al*.,2012).

Membrane CD23 is a functional trimer with three lectin domains that are connected to the membrane via a triple -helical coiled stalk . The affinity (*K*a) of human IgE for a single lectin domain is $10^6 – 10^7$ M^{-1}, however the avidity of human IgE for CD23 on the cell surface in its trimeric form is $10^8 – 10^9$ M^{-1} (Gould *et al.,* 2008). Two isoforms of CD23 have been identified, CD23a and CD23b, which differ by 7 and 6 amino acids in their N-terminal cytoplasmic regions respectively, and contain different signaling motifs that determine their functions . Human CD23a is expressed exclusively on antigen-activated B cells prior to differentiation into antibody-secreting plasma cells, and is involved in IgE antibody-dependent antigen endocytosis, processing and presentation, and the regulation of IgE synthesis and clearance. CD23b is expressed on a variety of

effector cells including B cells and monocytes/macrophages, following interleukin 4 (IL-4) stimulation (Yokota *et al.,*1992; Acharya *et al.,* 2010).

As all antibody classes, IgE is produced as secretory (sIgE) or membrane-bound (mIgE) isoforms. mIgE, together with the accessory proteins Igα and Igβ, constitutes the ε B cell receptor isotype (BCR), involved in antigen recognition and B cell differentiation (Batista.*et.al* 1996). In a cell-to-cell context, a functional interaction between mIgE and FcεRI has been reported that triggered receptor activation also in absence of the antigen. A truncated mIgE version containing Cε3 and Cε4 (but deprived of Cε2 and the Fabs) could also interact and activate FcεRI, underlining the importance of these domains in the IgE binding to the receptor (Vangelista .*et.al* 2005)

1.6 Role of IgE in Allergy

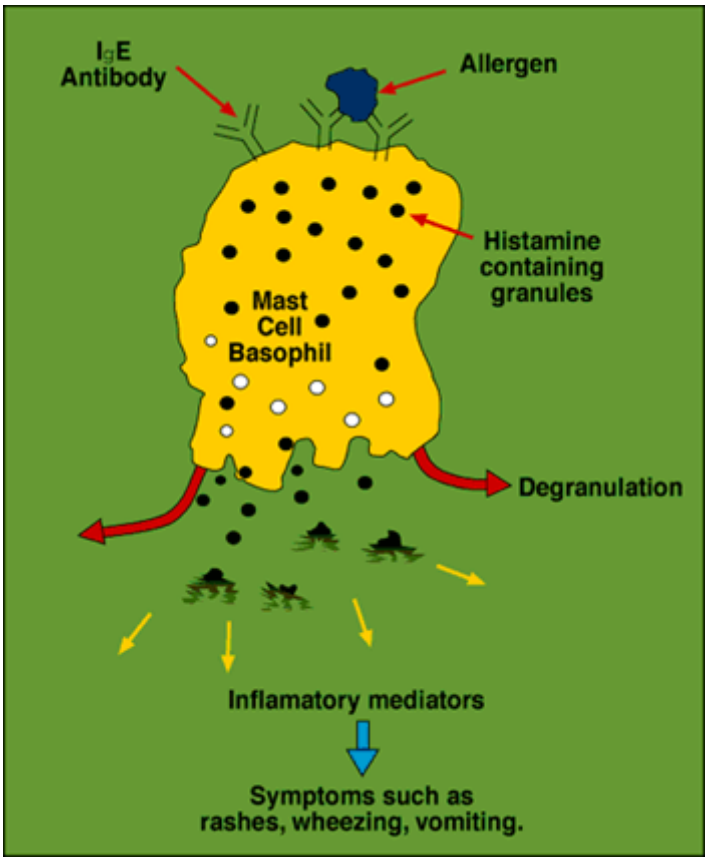


Figure.2 IgE mediated response

During an allergic manifestation, a powerful IgE-mediated inflammatory response is induced in response to common antigens such as dust or pollen allergens. In the first phase, called allergen sensitization, antigen presenting cells (APCs) such as DC and monocytes process and present the antigens to naïve T cells that differentiate into $CD4^+$ Th2 cells. Interaction between Th2

cells and B cells *via* major histocompatibility complex (MHC) class II and co-stimulatory molecules, such as CD40, induces B cells to undergo class-switch recombination from IgM or IgD to IgE (Gould *et.al* 2008). Differentiation into IgE-secreting plasma cells can also be induced by IL-4 and IL-13 produced by mast cells and basophils (Galli *et.al* 2012). As a result of allergen sensitization, allergen-specific IgE binds, *via* its Fc region, to the high affinity receptor expressed on the surface of mast cells and basophils, leaving its Fab regions available for future interaction with the allergen. Other cells known to express high-affinity receptors for IgE include basophils, Langerhans cells and activated monocytes. Production of allergen specific IgE-antibodies completes the immune response known as sensitization. On re-exposure, binding of the allergen to IgE orchestrates the immune system to initiate a more aggressive and rapid memory response. Cross-linking of a sufficient number of mast cell/basophil-bound IgE antibodies by allergen initiates a process of intra-cellular signaling, leading to degranulation of effector cells, with the release of mediators of inflammation. Mast cells attempt to sustain a fixed number of unoccupied high-affinity IgE receptors on their cell surface. IgE antibodies bind to these receptor sites, waiting for their specific allergen to be encountered (Galli *et.al* 2012).

The immune system's response to allergen exposure can be divided into two phases. The first is immediate hypersensitivity that occurs within 15 minutes of exposure to the allergen. The second, or late phase reaction, occurs 4-6 hours after the disappearance of the first phase symptoms and can last for days or even weeks. During the early phase reaction chemical mediators released by mast cells including histamine, prostaglandins, leukotrienes and thromboxane produce local tissue responses characteristic of an allergic reaction. In the respiratory tract for example, these include sneezing, oedema and mucus secretion, with vasodilatation in the nose, leading to nasal blockage, and bronchoconstriction in the lung, leading to wheezing. During the late phase reaction in the lung, cellular infiltration, fibrin deposition and tissue destruction resulting from the sustained allergic response lead to increased bronchial reactivity, oedema and further inflammatory cell recruitment. These observations suggest that IgE is instrumental in the immune system's response to allergens by virtue of its ability to trigger mast cell mediator release, leading directly to both the early and late phase reactions (Corry *et al.,* 1999; Chang ,2000; Hawrylowicz *et al*., 2010; Bax *et al*., 2012).

1.7 AllergoOncology: Directing IgE-Mediated Allergy towards Cancer

The emerging field of allergo-oncology represents a multi-disciplinary attempt to determine the relationship between cancer and IgE-mediated immunity, and to harness this relationship to develop active and passive immunotherapies for the treatment of cancer (Jensen-Jarolim *et al*., 2008). From1950s itself, experiments were carried out investigate for 'allergic responses' towards tumor transplants (Molomut *et al.,* 1955) The immunological observations were even termed as tumor allergy (Berdel *et al.,* 1956).Discussions pointed towards the biological relevance of tumor allergy for tumor progression(Schlitte *et al*., 1961; McCormick *et al*., 1971),but soon enough a negative association between allergy and cancer was announced(Ure et al., 1979). Numerous clinical surveys have shown that the atopic population has a decreased risk of malignancy and that a decreased prevalence of immediate hypersensitivity has been observed in cancer patients (Fisherman,1960; McKay. 1966; Ure, 1969; Meers, 1973; Alderson, 1974; Allegra *et al.,* 1976). Based on these clinical surveys, several epidemiological studies have investigated potential associations between allergy history and cancer risk with strong inverse associations reported in studies of pancreatic cancer, glioma, and childhood leukemia. IgE levels and atopy reactions in the skin of cancer patients were examined indicating that prevalence of atopy was decreased in cancer patients (Augustin *et al.,* 1971). Weekly injections of histamine and serotonin inhibited tumor growth in a transplant mouse model, pointing towards a possible role of anaphylactic reactions in tumor immunity (Jacobs *et al.,* 1972). Immunohistochemical study on the distribution of immunoglobulin classes in head and neck cancer revealed IgE antibodies to be the most abundant class, fixed in the cancer tissues on dispersed macrophage-like cells (shibashi *et al*.,2006; Fernandez-Acenero *et al*.,2000; Nielsen *et al*., 1999; Samoszuk *et al.,* 1997). From these studies it was clear that relationship between allergy and cancer is complex, and number of factors need to be considered such as reverse causality and/or treatment-related effects potentially influencing results (Van Hemelrijck *et al.,* 2010). Despite this, strong inverse associations have been consistently reported for a few specific tumor types, including cancers of the brain, pancreas, lymphatic and hematopoietic systems (Turner *et al.,* 2010). These studies points towards limited inverse associations and IgE here may have a natural surveillance function in malignancies.

1.8 Rationale behind use of IgE in therauptics

If IgE antibodies are directed against specific over expressed tumor-associated antigens (TAA) they could mediate the cell-to-cell association between tumor and effector cells, possibly resulting in ADCC and ADCP. The IgE isotype possess several unique properties which make them an attractive option for cancer therapy. They include:

Highest Determined affinity to receptors: The power of the IgE resides in its high affinity and specific binding with its receptors. The affinity of IgE for FcεRI(*K*a = 10^9 – 10^{11} M^{-1}) is by two to three orders of magnitude higher than that of IgG for their receptors, also the affinity of human IgE for CD23 on the cell surface in its trimeric form is *K*a = 10^8 – 10^9 M^{-1} which is equivalent to the affinity of IgG for FcγRI ,thus making IgE the only antibody class being strongly retained by effector cells in the absence of antigen. Hence, IgE engaged to FcεRI in tissues could be more effective in anti- tumor responses than IgG and its receptors. FcεRI-expressing monocytes exerted primarily ADCC towards tumor cells. Upon upregulation of FcεRI by preincubation with IgE specific for the ovarian tumor antigen folate receptor, an increase in ADCC was observed (Bracher *et al*., 2007; Karagiannis *et al*., 2007; Karagiannis *et al.,* 2008). Engagement of CD23b by IgE–antigen complexes promotes monocyte/macrophage activation, induces nitric oxide synthase (iNOS) and generates pro-inflammatory cytokines (Paul-Eugene *et al*., 1995; Mossalayi *et al.,* 1994). CD23b mediates IgE ADCP (Yokota *et al.,*1992) and may thereby, besides its control function for IgE production (Yu P *et al*., 1994), mediate tumor cell death. This function has been confirmed as a CD23–IgE complex-driven mechanism of engaging monocytes in ADCP of ovarian tumor cells upregulated by IL-4 (Karagiannis *et al.,* 2007).Thus IgE antibody by engaging antigens with cell surface receptors, FcεRI and CD23, activates several lines of effector cells against tumor cells in vitro and in vivo.

Expression of Receptors on Effector cells and Antigen presenting cells(APC): In humans, the 'classical' tetrameric FcεRI (αβγ2) is constitutively expressed on effector cells of anaphylaxis (i.e. mast cells and basophils), whereas a trimeric form of FcεRI (αγ2) is variably expressed on antigen-presenting cells such as monocytes, Langerhans cells and peripheral blood dendritic cells .FcεRI on antigen-presenting cells can deliver IgE-bound antigen into MHC class II antigen-presentation pathways(Novak *et al.,* 2001)

It is well established that solid tumors are associated with inflammatory responses involving the infiltration by not only B and T lymphocytes, neutrophils and natural killer cells, but also mast cells, macrophages and eosinophils expressing the IgE receptors(Brigati *et al.,* 2002)

Mast cells and Basophils:-Mast cells are the first immune cells to infiltrate the tumor microenvironment but their contribution to tumor growth and spread is controversial (Crivellato *et al.,* 2008) and dependent on the type of tumor. In some human cancers, such as breast and colorectal cancers the presence of mast cells has been associated with favorable clinical prognosis (Sinnamon *et a.,l* 2008), whereas Hodgkin's lymphoma and melanoma patients with higher numbers of mast cells in tumor lesions have a worse prognosis (Johansson *et al*., (2010). Remarkably, it has been proposed that even the different location of mast cells within the tumor microenvironment is prognostic, with a high intra-tumoral density being associated with a favorable prognosis and a high peri-tumoral density associated with a poor prognosis (Welsh *et al*., 2005). It has been proposed that mast cells can influence tumor angiogenesis, tumor invasion and contribute to the composition of the immune-suppressive tumor microenvironment. Preclinical studies suggested that blocking mast cell degranulation or depleting mast cells in some types of cancer might be an effective therapeutic strategy. However more studies are necessary to better understand whether mast cells can be therapeutically targeted in the tumor microenvironment to improve protective immune responses (Dalton *et al*., 2012).

The role of basophils in cancer is not completely clarified yet. They have been observed in the inflammatory infiltrate in experimental tumors. They are probably recruited by *in situ* expression of cytokines and chemokines and are considered to be associated with tumor regression (Di Carlo *et al*., 1998). In chronic myeloid leukemia basophils markers are used for diagnostic and prognostic evaluation (Valent *et al*., 2008).

Eosinophils: - Eosinophils are observed in the peri-tumoural infiltrate of several types of cancers, including hematological malignancies and solid tumours, and therby referred to as Tumor associated tissue eosinophilia (TATE)(Munitz and Levi-Schaffer, 2004). TATE has been suggested to represent a positive prognostic indicator in a number of tumours including colorectal carcinoma, oral and oesophageal squamous cell carcinoma, laryngeal carcinoma, pulmonary adenocarcinoma, and bladder carcinoma (Fernandez-Acenero *et al.,* 2000, Dorta *et al.,* 2002). In contrast they has also been associated with poor prognosis in Hodgkin's lymphoma (Von Wasielewski *et al.,* 2000) Eosinophils contain cytotoxic granule proteins, eosinophil peroxidase, cationic protein and

eosinophil-derived neurotoxin , which are able to induce tissue damage, enhancing local inflammation and immune response. Some of these eosinophil-derived cytotoxic mediators can induce tumor cell apoptosis and have been considered as potential cancer treatments(Huland *et al.,* 1992). Degranulated eosinophils have been detected in tumors following systemic administration of IL-2, suggesting that they are involved in tumoricidal activity. eosinophil infiltration into tumours in wild-type mice has been shown to be an early and persistent response (Cormier *et al.,* 2006). In addition, IL-5 transgenic mice, which express high eosinophil counts, demonstrated a significant reduction in tumour establishment and growth, which was correlated with a high level of eosinophil recruitment to the tumour and surrounding connective tissue (Simson *et al*., 2007). Taken together, these results indicate that a Th2-type response involving eosinophils is associated with tumour eradication in several animal models.

Macrophages and Monocytes: - Tumor-associated macrophages (TAM) are found in virtually all types of tumors and comprise more than 50% of the total tumor mass (Leek *et al.,* 1996). Tumor-associated macrophages activities are dependent on their polarization state: classical (M1; IFNα/LPS-dependent) or alternative (M2; IL-4/IL-13/IL-10-dependent)(Biswas *et al.,* 2011). In this regard, emerging therapeutics are now focusing on the repolarization of tumor-associated macrophages as a method to invoke their anti-tumor potential (Beatty *et al.,* 2011).

Blood monocytes are recruited to the tumor sites by chemokines and cytokines released by tumor cells and neighboring endothelial cells. They can be stimulated to either kill tumor cells and release angiostatic compounds, or, like mast cells, promote tumor growth and metastasis by producing angiogenic factors and matrix metalloproteases (Mantovani *et al*., 2002).

Antigen-Presenting Cells:- APC such as B lymphocytes, Langerhans cells and dendritic cells present in tumor infiltrates express CD23 and/or FcεRI .One of the mechanisms of immune escape by tumor cell is the defective differentiation and maturations of APC, with the consequent reduction of adaptive immune responses against tumor antigens. The presence of IgE bound to the surface of DCs *via* FcεRI interaction may increase the efficacy of antigen uptake and presentation by a 100-1000 fold, leading to an efficient activation of T cells that results in a powerful antitumor adaptive immune response(Luiten *et al.,* 1996; Luiten *et al.,* 1997).

Absence of Fc inhibitory receptor for IgE:- IgE antibodies lack inhibitory Fc receptors whereas IgG is subjected to the inhibitory receptor FcγRIIb, suggesting that IgE antibodies may escape, to some degree, the suppressive effects in the Tumor microenvironment(Woof *et al.,* 2004).

In contrast to IgE where serum concentrations compose only 0.02% of the total antibody population, IgG constitutes up to 85%, suggesting that a larger pool of endogenous IgG competitors for cell surface receptors could reduce the ADCC efficacy of a therapeutic IgG dose. Also Unlike IgGs, which have a serum half-life of 23 days, the half-life of unbound IgE immunoglobulins in serum is around 2 days, therefore, it is likely that repeated administration of IgE may improve the therapeutic results (Reali *et al*., 2001).

1.9 IgE Therapy based Experiments

IgE in passive immunotherapy

IgE in the passive immunotherapy of cancer offers several advantages over conventional IgG-based approaches, due to its high- and low-affinity receptors present on a broad spectrum of effector cells, its capacity for antigen uptake and presentation leading to a secondary immune response. IgE in serum is only 0.02% as compared to 85% in case of endogenous IgG suggesting that competitors for cell surface receptors could reduce the ADCC efficacy of a therapeutic IgG dose (Luiten *et al.,*1996). From this fact and its cytophilicity it may be expected that lower passive doses of IgE antibody preparations than necessary for IgG will be sufficient to achieve therapeutic effects on the targeted tumor (Leek *et al*., 1996). The binding of IgE to FcεRI and CD23 promotes several cell killing modes: i) pro-inflammatory mediators and cytokines released by mast cells and basophils recruit professional killer cells (such as neutrophils and eosinophils) on site; ii) antibody dependent cellular cytotoxicity (ADCC) causes target cell lysis through enzymes and cytokines release; and iii) antibody dependent cellular phagocytosis (ADCP) is mediated by macrophages and monocytes resident in the tumor microenvironment.

Murine Anti-gp36 IgE

Nagy and collaborators in early 1900s for the first time generated murine IgE antibodies targeting a glycoprotein (gp36) of the mouse mammary tumor virus (MMTV)(NAGY *et al*.,1991). The monoclonal IgE therapy was capable of preventing subcutaneous tumor development in 50% of the animals treated, but did not protect mice exposed to MMTV-negative mammary carcinoma cells (MA16/c).

Murine and Murine/Human Chimeric Anti-CCA IgE

A murine IgE(m30.6) targeting an antigenic determinant on the colorectal carcinoma cells was shown to transiently inhibit the growth of COLO 205 cells injected subcutaneously into SCID mice while both a mouse/human chimeric IgG1(ch30.6) and IgE(ch30.6) containing the variable region and corresponding human constant regions showed no effect (103). The lack of efficacy of ch30.6 IgE is not surprising since human IgE does not interact with murine FcεRI and hence anti-tumor effect was not to be seen (Mount *et al .,*1994).

Rat/Human Chimeric anti-Murine Ly-2 IgE

The antitumor effect of a rat/human chimeric IgE specific for the murine Ly-2 antigen has been explored. This antibody, originally constructed for evaluating antibody-mediated elimination of CD8-expressing target cells *in vivo* (Issacs *et a.,l* 1992), has been employed together with murine cytotoxic T cells (CTLs) redirected to recognize TAA in a non-major histocompatibility (non-MHC)-dependent manner. According to this strategy, CTLs have been stably transfected to express a chimeric FcεRI-α receptor. This receptor is composed by the extracellular domain of human FcεRI, the trans-membrane domain of human FcγRIIa and the intracellular human CD3-α signaling domain. C57BL/6 mice injected s.c. with syngeneic Ly-2-expressing E3 thymoma cells together with anti-Ly-2 IgE and FcεRI-α receptor-expressing CTLs showed a significant protection from tumor growth with 80% of survival, as compared to controls without anti-Ly-2 IgE (Kershaw *et al.,* 1996).

The same strategy was also used in immunosuppressed animals utilising primary human T cells retrovirally transduced with a chimeric FcεRI receptor linked to the cytoplasmic domains of the human co-stimulatory molecule CD28 in order to potentiate the activation signal of the CTL (Teng *et al.,* 2006). There was induction of IgE-mediated Ly-2^+ E3 thymoma cell lysis *in vitro* with production of immune-stimulatory cytokines such as IFN-γ and GM-CSF. Furthermore, adoptive transfer of engineered primary human T cells redirected toward E3 thymoma cells by anti-Ly-2 IgE resulted in *in vivo* anti-tumor activity with significant prolonged survival of treated Ly-2^+ tumor-bearing mice as compared to controls. These data demonstrated that primary human T cells expressing the chimeric IgE receptor could suppress tumour growth in the presence of a tumour-targeted IgE.

Murine/Human Chimeric Anti-Human FRα IgE

The potency of IgE interactions with effector cells and their receptors in tumor cell killing has been demonstrated using chimeric antibodies (MOv18 IgE and MOv18 IgG1) against an ovarian tumor-specific antigen, folate binding protein, expressed in 80% of ovarian cancers. The *in vivo* efficacy of MOv18 IgE and IgG1 has been compared in two separate human xenograft models of FRα-expressing ovarian carcinoma grown in immunodeficient mice. In the first model, SCID mice were challenged s.c. with FRα-expressing human ovarian carcinoma (IGROV1) cells. Following, i.v. administration of human peripheral blood mononuclear cells (PBMC), added as effector cells, was conducted in presence of either MOv18 IgE or MOv18 IgG1. MOv18 IgE with human PBMC gave significantly greater protection than PBMC alone, while MOv18 IgG1 with human PBMC offered no survival advantage. Furthermore, the use of monocyte-depleted PBMC *in vivo* resulted in a loss of the survival advantage conferred by MOv18 IgE. Flow cytometry analysis attested that MOv18 IgE-dependent tumor cell killing by human monocytes is mediated by two distinct pathways: ADCC *via* FcεRI and ADCP *via* CD23, both expressed on the surface of IL-4-activated monocytes. The involvement of human eosinophils as potent effector cells in MOv18 IgE Ab-dependent cytotoxicity *in vitro* has also been shown (Karagiannis *et al.,* 2003; Nagy E *et al.,* 1991; Kershaw *et al*., 1998).

Murine/Human Chimeric anti-Human MUC-1 IgE and anti-Human CD20

The human FcERIα transgenic mouse model has been employed to study a murine/human chimeric IgE mAb specific for the human epithelial antigen MUC-1. A modest inhibition of tumor growth was observed after peri-tumoral injection of the anti-hMUC-1 IgE in hMUC-1-expressing 4T1 tumor-bearing mice. However, the same tumor cells engineered to express an anti-hMUC-1 mouse IgE together with either MCP-1 (4T1.hMUC-1/MCP-1) or IL-5 (4T1.hMUC-1/IL-5), two chemoattractant cytokines, failed to grow. This result emphasizes the importance of antibody delivery to the tumor site and the presence of effector cells in the tumor microenvironment. Remarkably, mice that rejected 4T1.hMUC-1/MCP-1 and 4T1.hMUC-1/IL-5 also rejected subsequent (30 days later) injections of wild type 4T1 cells, suggesting the development of a memory immune response(Teo. *et al.,* 2012).

The same group produced a murine/human chimeric IgE antibody specific for the human B cell antigen CD20. The anti-hCD20 IgE was capable to drive ADCC by umbilical cord blood-purified mast cells and basophils against the OCI-Ly8 lymphoma B cell line. According to the authors, the

anti-hCD20 IgE activity *in vivo* was not studied because the significant levels of circulating antigen in a physiological model of lymphoma would have led to a high risk of anaphylaxis upon IgE-treatment (Teo *et al.,* 2012).

Human Anti-EGFR IgE

Two more variants can be added to the list of anti-tumor IgE, both engineered to target the human epidermal growth factor receptor (EGFR).Increased EGFR expression and activation and have been found in a variety of human tumors, EGFR constitutes a key target for cancer therapeutic approaches, including monoclonal antibodies. Spillner and colleagues focus their attention on the numerous anti-EGFR IgG antibodies to study the therapeutic potential of the human IgE isotype variant in tumor cell targeting. Proliferation and cytotoxicity assays proved both signal blocking and effector mediating capability by the anti-EGFR IgE mAbs. Interestingly, while phagocytosis remained nearly identical, cytotoxicity, with consequent tumor cell killing, increased up to 95% as compared to the IgG counterparts (Ford *et al.,* 2003).

Oral Mimotope Vaccination

An oral mimotope vaccination protocol has also been described with the aim of inducing tumour antigen-specific IgE antibodies (Riemer *et al.,* 2007). For this, epitope mimics, so-called mimotopes, for the epitope recognised by the anti-HER-2 antibody trastuzumab were developed. It resulted in the formation of serum IgE antibodies towards the HER-2 antigen, and anti-HER-2 IgE-sensitized effector cells were subsequently shown to mediate HER-2-expressing tumour cell lysis *in vitro* in an antibody-dependent cytotoxicity assay. These experiments indicated that directed and epitope-specific induction of IgE against tumour antigens is feasible with an oral mimotope vaccination regimen, and that these antibodies mediate anti-cancer effects *in vitro.*

1.10 IgE in Antitumor Adjuvanticity

A strategy was designed to redirect IgE-driven mechanism towards tumor. The utilization of IgE as an adjuvant for cancer immunotherapy is based on the hypothesis that tumor could be disguised as an intestinal parasite or an allergen aggregate, targeting IgE-FcεRI expressing on the effector cells within tumor microenvironment ,likely promoting a powerful inflammation with consequent immunological response at tumor site and restriction of tumor (Reali *et al.,* 2001).

IgE antibodies have been targeted on the surface of tumor cells by a three-step strategy consisting in the creation of an avidin bridge between a biotinylated TAA-specific antibody and a biotinylated monoclonal IgE (irrelevant specificity). C57BL/6 mice were injected s.c. with syngenic MC38-CEA-2 tumor cells, and two days later, mice were injected intraperitoneally (i.p.) with a biotinylated tumour-specific (anti-CEA-2) murine IgG antibody to target the tumour. The next day, mice were given avidin i.p. and, on day 4, mice received biotinylated anti-dinitrophenyl (DNP) murine IgE (or a biotinylated IgG antibody as control). IgE treatment (compared to treatment with IgG) caused both a delay in tumor development and a decrease in the rate of tumor growth with prolonged mice survival, conferring protection against subsequent challenges with untreated tumor cells. The Fc region of IgE should play a critical role in its efficacy, since heat inactivation of the biotinylated IgE prior to injection abrogated the anti-tumor effect. Depletion of eosinophils, $CD8^+$, or $CD4^+$ cells also abrogated the anti-tumor effect, demonstrating the requirement for these cell types in the IgE-mediated growth inhibition. These findings were confirmed under similar conditions in a more immunogenic tumor model, the syngeneic murine lymphoma RMA-Thy1.1. Furthermore, mice immunized with a cellular vaccine constituted by IgE-loaded, irradiated tumor cells were protected after tumor challenge with untreated tumor cell (Reali *et al.,* 2001). These results demonstrated that IgEs targeted on tumor cells not only possess a curative potential but also confer long-term antitumor immunity and that IgE-driven antitumor activity is not restricted to the activation of innate immunity effector mechanisms but also results from eosinophil-dependent priming of a T-cell-mediated adaptive immune response.

The adjuvant effect of IgE-coated tumor cells was later confirmed using a slightly different strategy (Nigro *et al.,* 2009). RMA tumor cells were first infected with modified vaccinia virus Ankara (MVA), a severely host-restricted viral vector, unable to multiply in human and in most mammalian cell lines (Sutter *et al.,* 1992). MVA infection substituted tumor cell irradiation and exploited the high immunogenicity of the vaccinia virus. RMA-infected cells were then conjugated with the hapten DNP and the haptenized cells coated with murine anti-DNP IgE and used to s.c. vaccinate C57BL/6 mice. After s.c. challenge with live RMA tumor cells, a strong anti-tumor effect was observed in these animals, as compared to mice vaccinated with haptenized tumour cells (not bearing IgE); however, when mice were vaccinated twice with haptenized tumor cells, an anti-tumor effect was also observed. This effect was similar to that observed in mice vaccinated only once with IgE-coated tumor cells, demonstrating the IgE adjuvanticity.

The adjuvant effect of IgE-coated tumour cells was later confirmed using a slightly different strategy (Nigro *et al.,* 2009). TS/A-LACK murine mammary adenocarcinoma cells(Benigni. et.al 2005) were first infected with modified Vaccinia virus Ankara (MVA) in order to avoid the need for irradiation of tumor cells prior to vaccination and to exploit the high immunogenicity of the Vaccinia virus. TS/A-LACK-infected cells were then conjugated with the hapten DNP and the haptenised cells coated with murine anti-DNP IgE and used to vaccinate BALB/c mice. Mice were vaccinated s.c. and then challenged s.c. with live TS/A-LACK tumor cells 15 days after vaccination. A strong anti-cancer effect was observed in these animals. Vaccination with haptenised tumor cells (not bearing IgE) did not induce an anti-tumor response; however, when mice were vaccinated twice with haptenised tumor cells, an anti-tumor effect was observed. This effect was similar to that of animals vaccinated once with IgE-coated tumor cells. The key role of FcεRI in the IgE anti-tumor adjuvant effect has been demonstrated using FcεRIα$^{-/-}$(Dombrowicz *et a.,l* 1993) and CD23$^{-/-}$ mice (Yu.P. *et al.,* 1994). Loss of tumor protection in FcεRIα $^{-/-}$ mice (but not in CD23$^{-/-}$ mice) confirmed the prominent role exerted by FcεRI in IgE anti-tumor adjuvanticity. In parallel, the use of a transgenic mouse h FcεRIα, in which the α chain of mouse FcεRIα is substituted with the human counterpart (Dombrowicz *et al.,* 1996), allowed us to demonstrate that also human IgE exerts anti-tumor adjuvanticity.

In the next studies to abolish the potential side effects of circulating IgE and eliminate the need for antigen specificity, they exploited the direct and functional interaction between membrane-bound IgE (mIgE) and FcεRI (Vangelista *et al.,* 2005). A truncated mIgE version (tmIgE) containing Cε3 and Cε4 (but deprived of Cε2 and the Fabs) has been engineered into a recombinant MVA (rMVA-tmIgE) and used to infect TS/A-LACK cells, with consequent transport of tmIgE to the surface of infected cells. Transgenic human FcεRIα mice were vaccinated s.c. and then challenged s.c. with live TS/A-LACK cells. Mice immunized with the rMVA-tmIgE cellular vaccine showed a significant attenuation of tumor growth compared to mice immunized with the control vaccine (not expressing tmIgE). This anti-tumor protection was completely lost when the cellular vaccine was administered to FcεRIα$^{-/-}$, thereby confirming the key role of FcεRI in IgE anti-tumour adjuvanticity(Nigro *et al.,* 2012).

AIM & OBJECTIVE

The primary aim of the work described in this thesis was to investigate if endogenous IgE has a active role in antitumor effect. Epidemilogical studies have suggested an inverse association between allergy and malignancies, therby directing our attention towards a possible role played by endogenous IgE in tumor surveillance

The objective of this thesis was to first setup *in vivo* tumor models in genetically modified mice for analysis of tumor growth .Initial data indicated KN1-Hyper transgenic(high IgE producer)mice to be completely resistant to tumor growth. To elucidate if indeed tumor protection was due to IgE, we generated a double mutant (high IgE producing/ FcεRIα KO).The principle objective here was to elucidate the role of endogenousIgE in tumor protection and the key role played by IgE:FcεRI interaction in antitumor protection

The initial part of thesis work details in setting up of tumor models of different cell lines, genetically modified mice and *in vitro* assays. Further in thesis we elucidate the role of endogenousIgE effect in *vivo* and *in vitro* experiments. In subsequent experiments we investigate that indeed IgE is involved in tumor suppression and is mediated by its interaction with high affinity receptor FcεRI, by generation of new transgenic mice

These studies have been designed to evaluate for immune response generated endogenously and the possibility to detect presence of tumor-specific IgE for a potent antitumor effect.

Material and Methods

Cell Culture Maintenance

Tissue culture was performed under sterile conditions in a laminar flow hood using aseptic technique. Cells were grown in a humidified atmosphere at 37˚C in 5% CO2 in a CO2 air-jacketed incubator. All cell culture media, unless otherwise specified, were supplemented with 10% (v/v) foetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin (PenStrep; standard additives).

Cell lines

TS/A-LACK :- (Leishmania receptor for activated C kinase) is a H-2d mammary adenocarcinoma cell line of BALB/c origin, provided by Dr. A. Mondino (San Raffaele Scientific Institute, Milan, Italy), maintained in RPMI 1640 medium supplemented with 100µg/mlgeneticin(G418:Calbiochem),10%(v/v) foetal calf serum (FCS)(Euroclone),1% of penicillin/streptomycin(GIBCO)

N2C:-The HER-2/neu expressing N2C primary mammary carcinoma cell lines were derived from female BALB-neuT mice,kindly provided by Dr. Mario .P. Colombo (Instituto Nazionale Tumori) maintained in DMEM(Life Technologies) supplemented with10%(v/v) foetal calf serum (FCS)(Euroclone),1% of penicillin/streptomycin(GIBCO)

RBL-2H3:-is a Rat Basophilic Cell line (Kulczycki Jr,A &Metzger,H 1974) maintained in DMEM(Life Technologies),10%(v/v) foetal calf serum (FCS)(Euroclone),1% of penicillin/streptomycin(GIBCO)

Mice

Hyper IgE(KN1): Kindly provided by Dr. Gernot Achatz, these mice are maintained and bred in animal facility and are in BALB/c background. This mouse strain expresses a chimeric ε-γ1 BCR, consisting of extracellular domains of the ε gene and transmembrane and cytoplasmic domains of the γ1 gene, influencing of γ1-mediated signaling of mIgE bearing B cells.They show an increased serum IgE levels(Gertrude-Achatz-Straussberger *et a.,l* 2008)

IgE knock out : These mice were also kindly provided by Dr.Gernot Achatz, maintained and bred in animal facility and are in BALB/c background. IgE knock out transgenic mice carried mutations in transmembrane and cytoplasmic domains of IgE in the germline ε gene, This was achieved using the gene-targeting technique in embryonic stem (ES) cells. Serum IgE was reduced by 94-98% in these mice(Gernot Achatz *et al.,* 1997)

FcεRI knock out : This transgenic mice was provided by Dr. Dombrowicz,maintained and bred in animal facility. They are in BALB/C background. This transgenic mice is obtained by knockdown of part of FcERI receptor gene for by disrupting 4^{th} exon by neo cassette insertion through homologous recombination. The deletion is in the α-chain thereby binding with IgE is abolished(Dombrowicz *et al.,* 1993).

HER-2/neu Transgenic : These Transgenic mice are bred and maintained in animal facility of Dr.M.P Colombo in Tumori Institute. A transgenic CD1 random-bred breeder male mouse carrying the mutated rat HER-2/neu oncogene driven by the MMTV promoter was mated with BALB/c females($H\text{-}2^{d}$). The progeny was screened by PCR. Transgene-carrying males were backcrossed with BALB/c females and HER-2/neu^{+} BALB/c mice(BALB-neuT) were used in this experiment(Katia Boggio *et al.,* 1998).

CD-23 (FcεRII) knock out : This transgenic mice bred and maintained in animal facility and obtained by confirmed disruption of FcεRIIα gene by neomycin cassette insertion through homologous recombination.(Yu P *et al.,*1994)

Wild Type : BALB/c mice (female 8 week of age) were purchased from Harlan Laboratories.

For experiments, five mice were considered in each group.

Vaccination and Tumor Challenge :

Immunizations were performed on five mice for each group by s.c. administration of irradiated TS/A lack cells(10,000 rad) (10^5 cells/mouse) in 0.2ml PBS at the base of the tail. Two weeks after immunization, mice were challenged by s.c. injection in the left flank with 2×10^5 TS/A-LACK cells in0.2ml PBS. Tumor growth was monitored at 1–3 day intervals by measuring tumor volume with a caliper. Tumor volume, V, was calculated by the equation $4/3\ \pi\times r_1\times r_2\times r_3$ where r_1 is longitudinal radius, r_2 is the lateral radius, and r_3 is the thickness of tumors protruding from the surface of normal skin (Gasparri *et al.*,1999)

N2C Tumor Challenge and Evaluation of Tumor growth:

Mice were challenged by s.c. injection in the left flank with 6×10^5 N2Ccells in 0.2 ml PBS. Tumor growth was monitored at 1–3 day intervals by measuring tumor volume with a caliper. Tumor volume, V, was calculated by the equation $4/3\ \pi\times r_1\times r_2\times r_3$ where r_1 is longitudinal radius, r_2 is the lateral radius, and r_3 is the thickness of tumors protruding from the surface of normal skin (Gasparri *et al.,*1999)

IgE mediated Release Assay:

Plastic-adherent RBL-2H3 cells (8×10^4 cells/well) were incubated in DMEM with 100 ng mouse anti-DNP IgE mAb (or anti-NIP human IgE mAb) for 2hours at 37°C. Cells were then washed and incubated in triggering buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6mM glucose, 1mM CaCl2, 0.4mM MgCl2, 0.1% BSA (pH 7.2)) with 100 ng human serum albumin (HSA)-DNP (Sigma-Aldrich) or NIP-BSA (Biosearch Technologies) for1hat 37°C.

Alternatively, Serum incubated tumor cells for 1hour at 4° C are taken in triggering buffer and loaded on to RBL- 2H3 cells. Plates were centrifuged 5 min at 2000 rpm and incubated for 1hour at 37°C. The release of β-hexosaminidase by RBL-2H3 was detected in the cell supernatant. Supernatants were transferred to a new plate and 1 mM p-nitrophenyl-N-acetyl in 0.1 M citrate buffer (pH 6.2) was added, followed by a 150 minutes of incubation at 37°C. The reaction was stopped using 0.1M stop solution (0.1M Na2CO3, 0.1 M NaHCO3, (pH 10.0)), and the absorbance was read at 405 nm. Negative control was the supernatant of nonstimulated cells. Positive control was the supernatant of IgE-sensitized RBL-2H3 cells stimulated with 100 ng HSA-DNP The positive

control represented 60% of the total hexosaminidase content obtained from cell lysis with 0.1% Triton X-100. The results are calculated as percentage of total content using the formula

$= 100 \times (A_{405}$ sample $\times A_{405}$ negative control$)/(A_{405}$ β-hexosaminidase content $\times A_{405}$ negative control).

CD-8 depletion Studies:

Hyper IgE mice were injected with 200 µg of anti-CD8 ascites before to tumor cell challenge on days -2,-4 and -7 and 14 days post Tumor cell challenge. The CD8 depletion was monitored by immunofluorescence study on whole blood cells.

FACs Analysis:

To confirm for CD8 depletion,200µl of whole blood was collected by venipuncture in heparinised tubes. The samples were incubated with 2-3ml of ACK lysis buffer for 2 minutes followed by PBS wash. They were incubated with Fluorescent protein–labeled mAbs against CD8(Cy-Chrome anti-mouseCD8a(ly-2)(clone 53-6.7) (from PharMingen, San Diego, CA).

For Experiments in Tumori Institute:

Bone Marrow Transplantation Experiment :

Seven- to eight-week-old mice were lethally γ-irradiated with 1,000 cGy and BMT performed as previously described .BMT engraftment was checked by staining with FITC-conjugated Kd and PE-conjugated Kb (from BD Biosciences) and subsequent fluorescence-activated cell sorting analysis . Mice were monitored for tumor development. Tumors were measured with a caliper once a week, and tumor volume is calculated as $d^2 \times D$, where d is the smaller diameter and D is the longer one.

FACs Analysis:

PBMCs were withdrawn from the retro-orbital sinus at 4 and 8 week after BMT and were stained with with FITC-conjugated mouse anti-mouse H-2Kb and PE-conjugated mouse anti-mouse H-2Kd (Cederlane,Hornby,Ontario,Canada)

DNA expression vectors and vaccination :

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequence for the ECD and

that for the ECD and TM domain of mutated r-p185 were generated from the PCR product using the primers,

39-CGCAAGCTTCATCATGGAGCTGGC-59 and

39-CGGAATTCGGGCTGGCTCTCTGCTC-59

and the primers 39-CGCAAGCTTCATGGAGCTGGC-59 and

39-ATGAATTCTTTCCGCATCGTGTACTTCTTCCGG-59, respectively, as previously described (Amici,A *et al.,*1998). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the two plasmids used in this work (ECD and ECD-TM plasmids). The pCMVb (Clontech Laboratories, Palo Alto, CA) coding for b-galactosidase was used as a control plasmid (b-gal plasmid). *Escherichia coli* strain DH5a was transformed with ECD, ECD-TM, and b-gal plasmids and then grown in Luria-Bertani medium (Sigma, St. Louis, MO) . Large-scale preparation of the plasmids was conducted by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth,CA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml, and stored in aliquots at 220°C for subsequentuse in immunization protocols. Plasmids (50 ug/injection) were injected into the quadriceps muscle through a 28-gauge needle syringe. BALB/c mice were immunized 21 and 7 days before tumor challenge (day 0), starting at the 10th wk of age. BALB-neuT mice were immunized at the 14^{th} wk of age.

RESULTS

3. Raising of Endogenous Antitumor IgE

It has been well summarized in review articles [Jacobs *et al.,* 1972; Allegra *et al.,* 1976] about the epidemiological studies examining the relation between allergy and cancer. Studies were partially inspired by largely debated epidemiological studies proposing a role for allergic conditions in decreasing the incidence of cancer (i.e., the presence of high IgE serum levels would contribute to the prevention of cancer development). So to determine if intrinsically IgE has a role in Tumor mortality, in-vivo experiments were setup making use of Transgenic Mice models with two different tumor cell line models, low immunogenic TS/A-LACK and highly immunogenic N2C cell line.

3.1 TS/A-LACK Tumor Model

3.1.1 Determination of in vivo anti-tumor power of Endogenous IgE against TS/A-LACK cell line

To detect the role of endogenous IgE response to tumor *in vivo, primary* experiment was set-up using transgenic mice where-in the IgE gene was either knocked off (IgE *knock out*),or engineered for high IgE production(KN1-HyperIgE)and wild type mice as controls. TS/A-LACK (TS/A-Leishmania receptor for activated C kinase (LACK) mammary adenocarcinoma of BALB/c origin)(Benigni *et al*.2005)cell line was considered for the experiment(Figure .3)Mice were challenged s.c on the left flank with 2×10^5 cells in 0.2ml of PBS. Subsequently the growth of tumor was monitored up to 30 days post tumor cells challenge or till tumor size exceeded above $500mm^{3.}$ From the tumor growth graph, we observe that in IgE KO group the tumor growth was highest followed by WT group. There is significant decrease in KN1-HyperIgE group indicating an antitumor role.

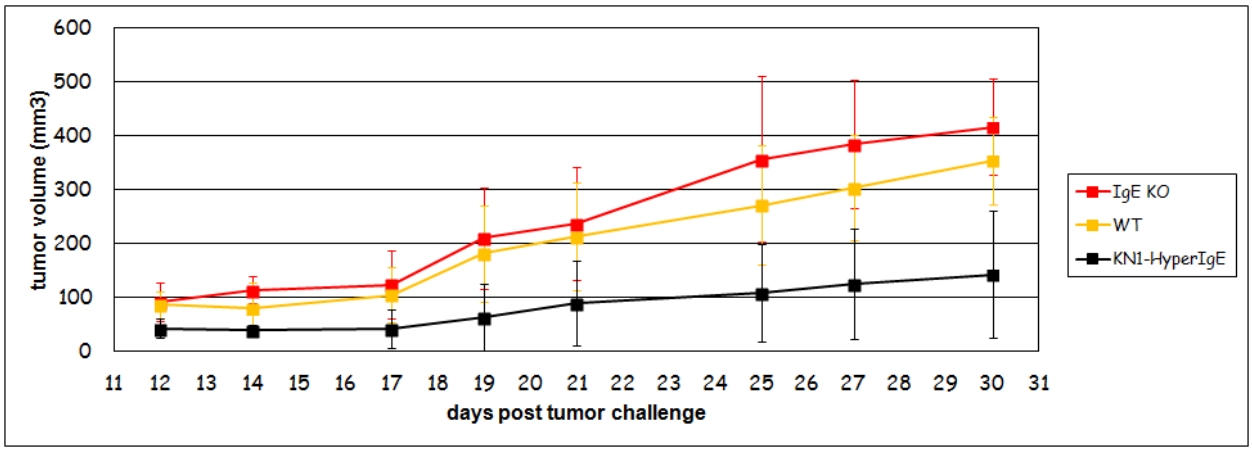


Figure .3 Anti-tumor responses in mice with altering levels of IgE production

IgE *knock-out*(KO)(blue line), wild type (green line) and KN1-HyperIgE (yellow line) were challenged by s.c injection in the left flank with 2×10^5 TS/A-LACK cells in 0.2ml of PBS.Tumor growth is considerably reduced in mice with high IgE production(KN1). (Values are mean ± SEM)

3.1.2 Immunization for a complete anti-tumor response

To achieve a better anti-tumor response *in vivo*, mice were immunized with irradiated TS/A-LACK 15 days prior to live tumor cells challenge .As experimental control, mice were also directly challenged with live TS/A-LACK cells without immunization. Mice were monitored for tumor growth up to 30 days post challenge or till tumor size exceeded above $500mm^3$ (Figure.4). Blood was collected before immunization, tumor cell challenge and before the end of experiment and serum was used for in-vitro assay. We observe that with or without immunization there is no protection seen in IgE ko and wild type groups but complete tumor protection with immunization and partial protection without immunization for KN1-Hyper IgE group indicating by vaccinating there was establishment of tumor specific-IgE memory cells.

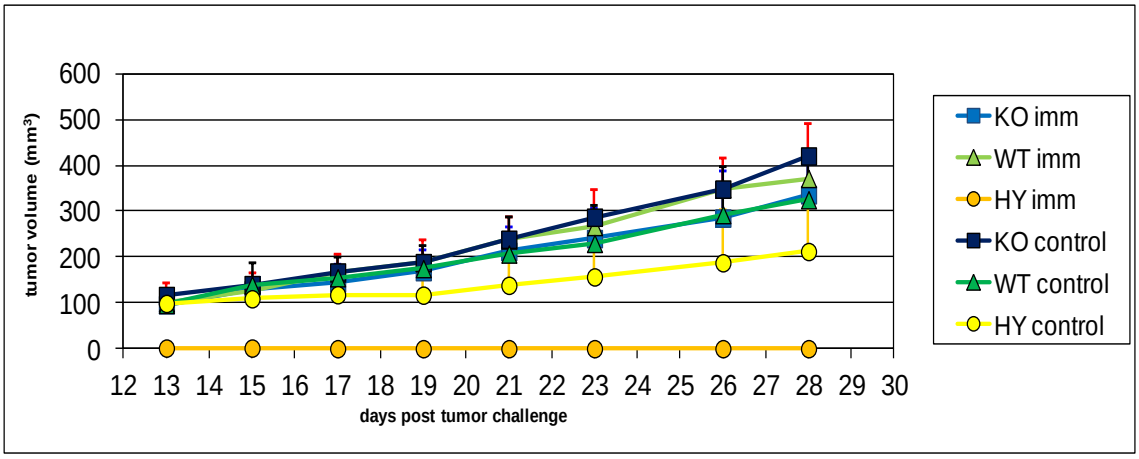


Figure.4 Anti-tumor protection against TS/A-LACK cells

Immunizations were performed on five mice for each group by s.c. administration of irradiated TS/A- LACK cells (10,000 rad) (10^5 cells/mouse) in 0.2ml PBS at the base of the tail. Following 15 days of immunization mice were challenged by s.c injection in the left flank with 2×10^5 TS/A-LACK cells in 0.2ml of PBS.

Transgenic Mice with high production of KN1-HyperIgE immunized group had completely suppressed tumor growth compared to IgE KO and *wild type* groups with or without immunization, though in non-immunized KN1-HyperIgE group there was considerably reduced tumor growth observed. (Values are mean ± SEM)

3.1.3 Subsequent Tumor rejection in immunized KN1-HyperIgE mice

The mice from the immunized KN1-HyperIgE group which were tumor free, upon subsequent challenge with TS/A-LACK were able to suppress tumor growth indicating the establishment of IgE memory cells against the tumor.

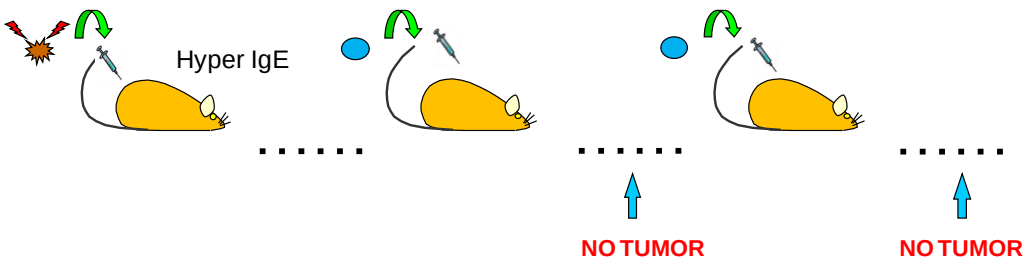


Figure 5. KN1-HyperIgE mice on subsequent tumor challenge after immunization were able to reject tumor growth.

Immunized KN1-HyperIgEmice which were tumor free upon first Challenge with TS/A-LACK cells are subsequently challenged second time with 2×10^5 TS/A-LACK cells in 0.2ml of PBS. Mice were completely protected against tumor growth.

3.1.4 In-vitro Characterization for production of Tumor specific IgE in mice determined by functionality assay

To evaluate if there is tumor specific IgE produced in mice challenged *in vivo* with tumor cells, a functionality test which triggers IgE-mediated mediator release was performed. When tumor cells are incubated with serum (containing antibodies) and loaded on to rat basophilic cell line RBL-2H3, expressing FcεRI, degranulation and mediator release are triggered via FcERI cross linking. Serum collected from mice of *in vivo* experiments are first incubated with TS/A-LACK tumor cells 2×10^5 for 1hr at 4° C and then put in contact with RBL-2H3 cells. Alternatively, RBL- 2H3 cells were also put in contact with TS/A-LACK tumor cells as control. The release of β-hexosaminidase by RBL-2H3 was detected in the supernatant. The 100% release positive control was obtained by stimulating RBL-2H3 with mIgE cross linked with soluble anti-IgE DNP-HSA,nonstimulated RBL-2H3 cells was used as control. Values are mean ± SD of three determinations.

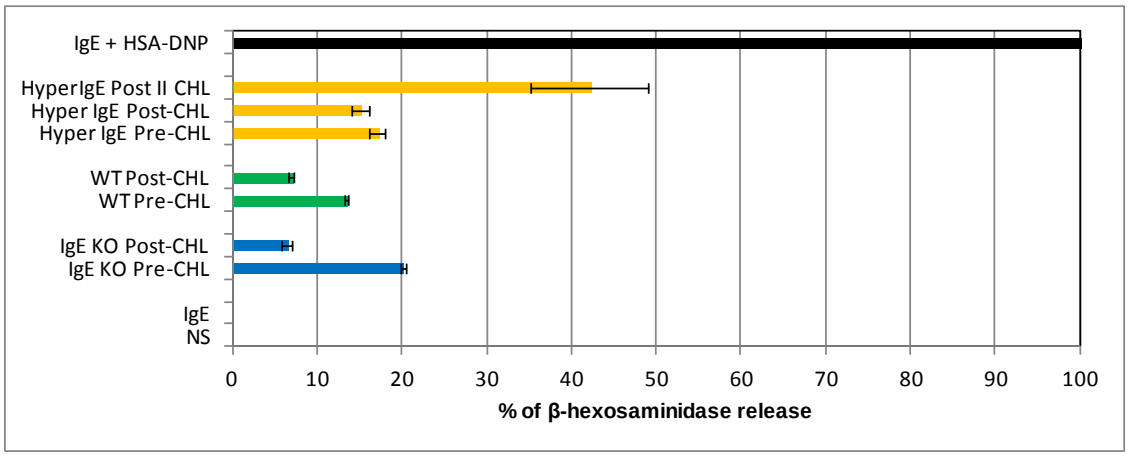


Figure .6 IgE mediated β-hexosaminidase release assay

RBL-2H3 cells in contact with serum(incubated with TS/A-LACK tumor cell) or as control just TS/A-LACK cells.100% of release corresponds to release obtained by lysing cells with 0.1% Triton X-100. Positive control consists of anti-DNP soluble IgE sensitized RBL-2H3 cells cross-linked with HAS-DNP (IgE+HSA-DNP).Negative controls: nonstimulated RBL-2H3 cells (NS):anti-DNP soluble IgE loaded to RBL-2H3 cells in absence of cross-linker HSA-DNP.Values are mean ± SD of three determinations

It was observed from these experiments IgE has a role in tumor suppression. Whereas tumor was able to grow in IgE *knock out* and *wild type* groups with or without immunization, there was complete tumor protection in immunized KN1-HyperIgE mice and considerable decrease in Tumor

growth in mice from this group which were not immunized. The tumor free KN1-HyperIgE group which on repeated tumor challenge was still tumor free pointing towards establishment of tumor specific IgE memory cells.

For detection of tumor specific IgE, two challenges were required after immunization. Though with the first challenge there was no difference in IgE mediated specific release, there is a significant increase after the subsequent challenge indicating towards establishment of IgE memory cells against the tumor. Moving ahead to improve the *in vitro* detection and to test this anti-tumor effect, a different tumor model was selected.

3.2 N2C Tumor Model

The HER-2/neu overexpressing N2C primary mammary carcinoma cell lines were derived from female BALB-neuT transgenic mice which spontaneously develop tumor (Sangaletti *et al.*,2003).HER-2/neu,a member of epidermal growth factor receptor family, encodes 185-kDa protein that is a transmembrane tyrosine kinase(Schechter et al.,1984)(Bargmann *et al.,* 1986).Over expression of neu is frequently observed in many cancers related to breast, ovary, lung and bladder.(The HER-2/neu oncoprotein is considered to be a tumor-associated antigen (Disis *et al.,*1997)).

3.2.1 Determination of cell population required for induction of tumor growth

Initially, Experiments were set to determine the minimal number of cells required for 100% growth of tumor. Transgenic mice, IgE *knock out* and KN1-HyperIgE mice were selected to set the cell count parameter. Mice were injected with different cell count and observed for tumor growth in both IgE *knock out* and KN1-HyperIgE transgenic (Figure 7a and 7b). In IgE *knock out* mice the tumor develops starting from 2×10^5 and increased with dose and at high dose of 6×10^5 cells it developed tumor of size 500 mm^3 in volume. In groups of KN1-HyperIgE mice, the doses at 2 and 4 $\times10^5$ cell count there was no tumor growth observed but in group challenged with 6×10^5 cells there was initiation followed by regression of tumor growth. So therefore 6×10^5 cells were set as the minimum required cells for further experiments.

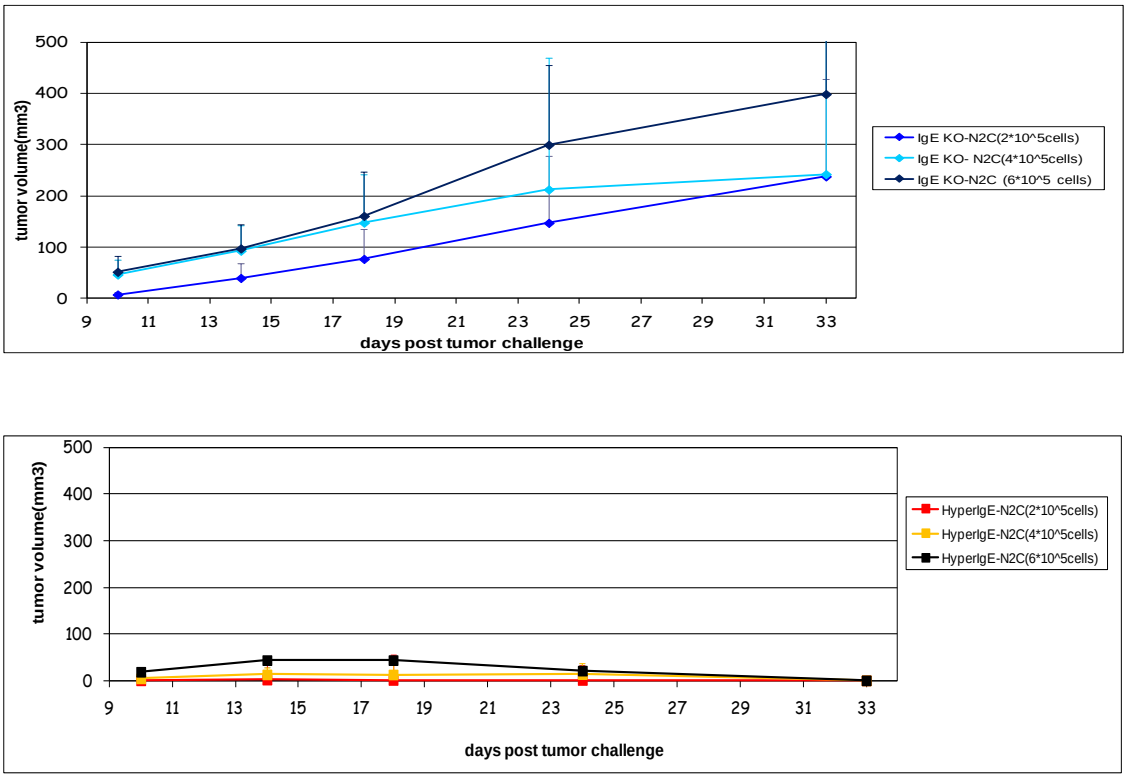


Figure 7 ; N2C cells required for induction of tumor growth

7a.) IgE KO(knock out) mice groups by s.c injection in the left flank with different dose of N2C(2×10^5, 4×10^5, 6×10^5) cells in 0.2ml of PBS.

7b.) KN1-HyperIgE mice groups by s.c injection in the left flank with different dose of N2C(2×10^5, 4×10^5, 6×10^5) cells in 0.2ml of PBS. Based on tumor growth seen in both the groups, 6×10^5 was considered for further experiments.

3.2.2 – Determination of in vivo antitumor activity with N2C tumor cells

To check the role of endogenous IgE towards tumor growth *in vivo,* experiment was set-up using transgenic mice IgE KO, KN1-HyperIgE and CD-23 KO(knock out for FcER II)(Figure .8). CD-23 KO mice was selected based on results from previous exogenous IgE studies in our laboratory(Nigro.et.al.2009) where it was demonstrated that CD-23 knock out (KO) had no role in IgE mediated antitumor immunity and behaved in similar manner to *wild type* group in tumor development. Mice were challenged s.c on the left flank with N2C cells 6×10^5 cells in 0.2ml of PBS. Subsequently the growth of tumor was monitored up to 30 days post tumor cells challenge or till tumor size exceeded above $500mm^{3.}$ In IgE *knock out* and CD-23 *knock out* group tumor growth is observed but complete tumor protection for KN1-HyperIgE group. These tumor free mice on subsequent N2C cell challenge there was no growth of tumor observed.

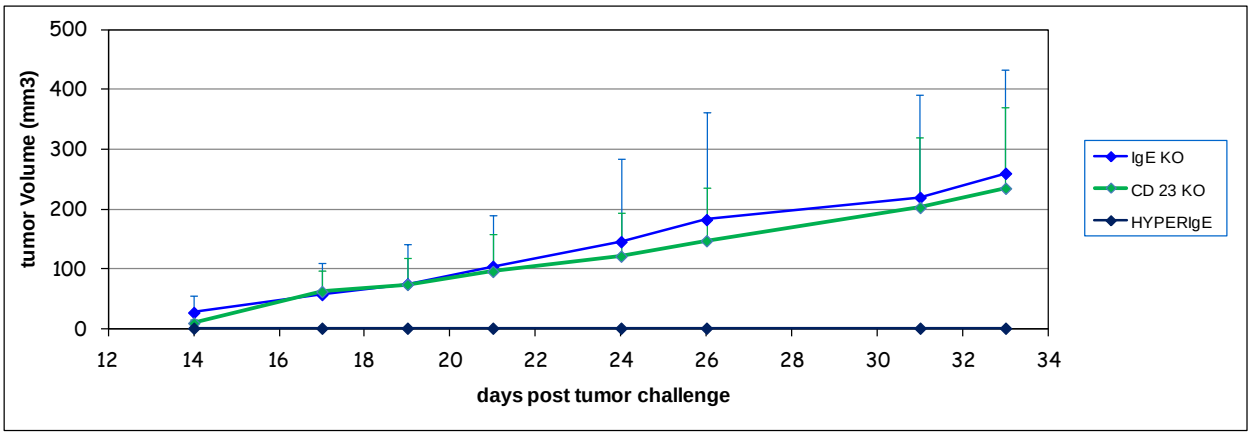


Figure .8 Anti-tumor protection against N2C cells

IgE *knock-out*(KO)(blue line), CD-23 *knock out* (KO) (green line) and KN1-Hyper IgE (yellow line) were challenged by s.c injection in the left flank with 6×10^5 TS/A-LACK cells in 0.2ml of PBS. Tumor growth is completely suppressed in group with KN1-Hyper IgE mice. Upon subsequent challenge on KN1-HyperIgE mice group which were tumor free, there was no growth observed, indicating towards tumor memory.(values are in mean±SEM)

3.2.3 In-vitro Characterization for production of N2C tumor specific IgE in mice determined by functionality assay

To check if there is production of tumor specific IgE produced in mice challenged *in vivo* with N2C cells, a functionality test which triggers IgE-mediated mediator release was performed. When N2C cells incubated with serum (containing antibodies) are loaded on to rat basophilic cell line RBL-2H3, expressing FcεRI, degranulation and mediator release are triggered via FcERI cross linking. Serum collected from mice of *in vivo* experiments are first incubated with N2C cells 2×10^5 for 1hr at 4° C and then put in contact with RBL-2H3 cells. Alternatively, RBL- 2H3 cells were also put in contact with N2C tumor cells as control (Figure 9). The release of β-hexosaminidase by RBL-2H3 was detected in the supernatant. The 100% release positive control was obtained by stimulating RBL-2H3 with mIgE cross linked with soluble anti-IgE DNP -HSA, nonstimulated RBL-2H3 cells was used as control. The results show that there is no tumor specific-IgE in IgE *knock out* and CD-23 *knock out* groups, whereas in KN1-HyperIgE tumor specific IgE is detected and its detection is increased post 30 days of tumor challenge and on subsequent tumor challenge. Values are mean ± SD of three determinations.

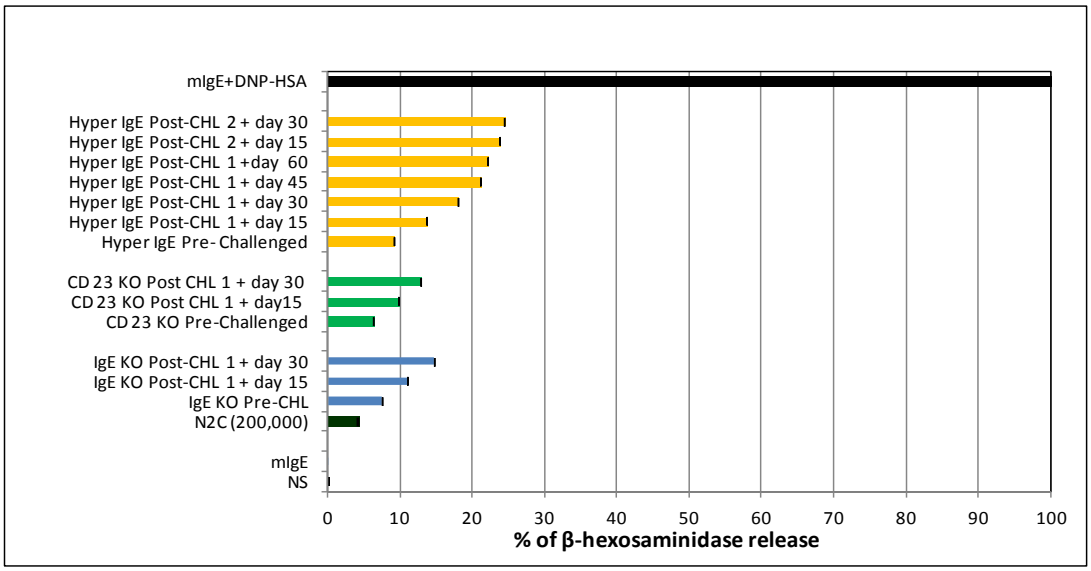


Figure .9 IgE mediated β-hexosaminidase release assay

There is specific increase from serum samples of KN1-Hyper IgE group and the release increases with serum collected after subsequent challenges indicating towards production of IgE memory cells. The detection can be seen only after 30 days post tumor challenge and increases with time.

RBL-2H3 cells in contact with serum(incubated with N2Ccells) or as control just N2C cells.100% of release corresponds to release obtained by lysing cells with 0.1% Triton X-100. Positive control consists of anti-DNP soluble IgE sensitized RBL-2H3 cells cross-linked with HAS-DNP (IgE+HSA-DNP).Negative controls: nonstimulated RBL-2H3 cells (NS):anti-DNP soluble IgE loaded to RBL-2H3 cells in absence of cross-linker HSA-DNP. Values are mean ± SD of three determinations.

It was evident from the primary experiment with N2C cells, that immunization protocol was not necessary as in KN1-HyperIgE mice group the tumor growth was regressed after initiation or suppression of tumor growth was observed, whereas in other group the tumor was able to grow. Even the in vitro detection of tumor specific IgE was increased. In this study, KN1-HyperIgE mice not developing tumor and increase in specific IgE of *in vitro* results can be due to N2C cells expressing HER-2/neu on its surface which are captured by high amount of IgE produced in KN1-HyperIgE mice.

These preliminary results pointed towards IgE behind tumor protection. To validate these results of role of IgE, we planned to investigate further through two models

The first study involved performing Bone Marrow Transplantation of KN1-HyperIgE mice. The N2C cells used in previous experiments were derived from spontaneous tumor developing HER-2/neu mice. Bone Marrow cells from KN1-HyperIgE were injected to these mice which develops tumors spontaneously starting by 10 weeks of age and die at 33 weeks of IgE. The aim of this experiment being to determine if bone marrow cells from KN1-HyperIgE are able to elicit an antitumor potency or delay tumor growth in these spontaneous tumor developing mice.

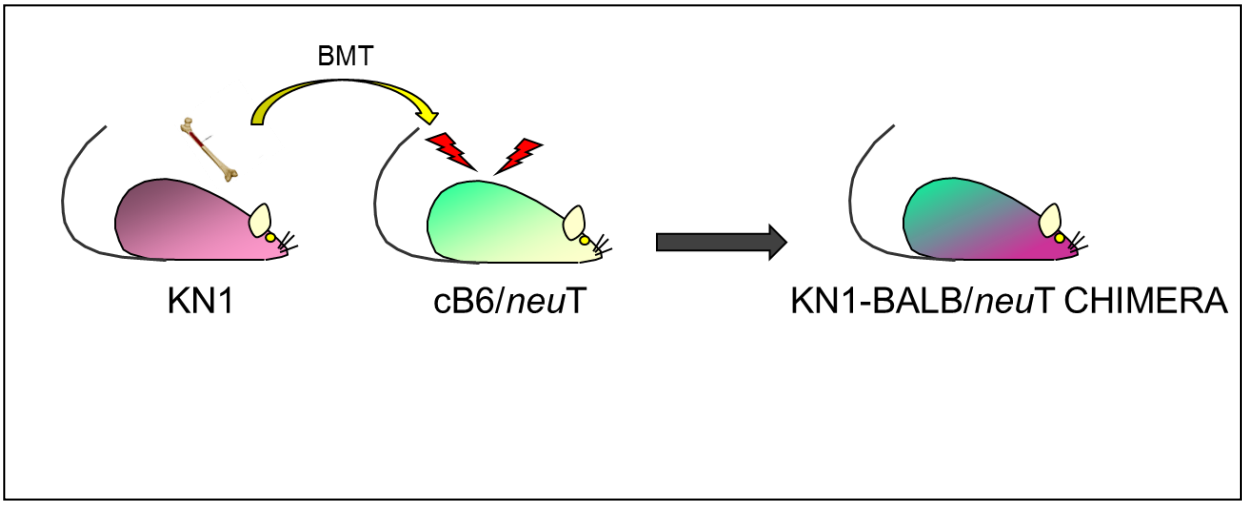


Figure 10. Does IgE establish tumor immunity in spontaneous Tumor developing mice?

In the second model, to validate that indeed endogenous IgE is involved in anti-tumor protection in-*vivo,*we planned to breed KN1-HyperIgE mice with FcεRIα *knock out* mice so as to generate a Double mutant transgenic mice which is high IgE producer but lacking in its receptor FcεRI. So when challenged with tumor if these mice develop tumor then it will be a indirect demonstration of role of IgE and prominence of receptor FcεRI's interaction with IgE as the mechanism involved in antitumor potency seen in KN1-HyperIgE mice.

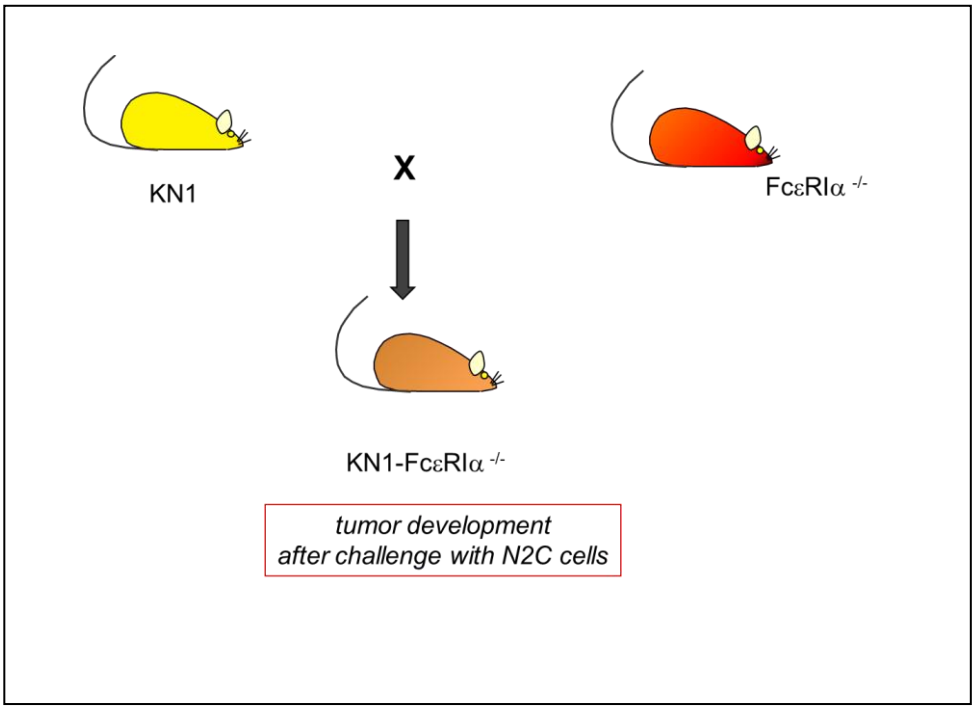


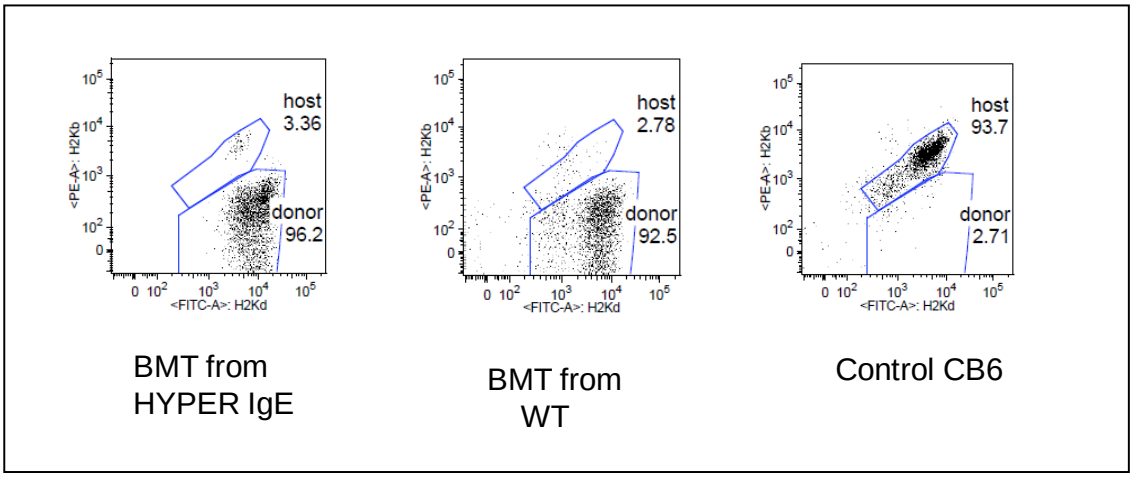
Figure 11. Indirect mechanism to study endogenous IgE's role in anti-tumor protection

3.3.1 Does Endogenous IgE has the power to induct an anti-tumor effect in spontaneous tumor bearing mice

The Her-2/neu gene encodes p185 tyrosine kinase growth factor receptor homologous to other members of epidermal growth factor receptor family (Bargmann *et.al* 1986).The involvement of over expressed and p185 in the initiation and progression of breast carcinogenesis makes it an interesting target for therapy (Hung *et al.,* 1999).Making use of Her-2/neu transgenic mice (Sangaletti *et al.,* 2003) in animal facility in Tumori Instituto. HER-2/neu transgenic CD1 mice is mated with BALB/c to obtain BALB-NeuT mice, these transgenic mice carry activated rat Her-2/neu oncogene. They start to develop multiple mammary tumors that progressively involve all 10 glands by 10 weeks. The N2C tumor cells used in previous study originated from these transgenic mice.

KN1-HyperIgE mice were able to resist tumor growth against N2C cells even without immunization. To evaluate if Bone-marrow cells from Hyper IgE mice when transplanted are able to elicit anti-tumor effect on these spontaneously tumor developing HER-2/neu mice experiments were set up. Seven-to eight week old mice were lethally γ-irradiated with 1,000 cGy and Bone Marrow cells injected from 8-10 week old KN1-HyperIgE. The engraftment was checked by staining with FITC-conjugated Kd and PE-conjugated kb and subsequent fluorescence-activated cell sorting analysis (Figure 12a). As a control for experiments Bone marrow cells from Wild type were used. Since BMT cells from KN1-HyperIge mice (BALB/c background) was injected on to HER-2/neuT mice(C57BL/6 background), through FACs we were able to monitor for immune cells engraftment from donor cells.It was evident that there was no antitumor protection elicited by KN1-HyperIgE BMT cells on HER-2/neuT mice. The tumor growth of WT BMT was comparable to KN1 BMT group.

12a.



12a. Fluorescence-activated cell sorting analysis- PBMCs were withdrawn from the retro-orbital sinus at 4 and 8 week after BMT and stained with FITC-conjugated mouse anti-mose H-2Kb and PE-conjugated mouse anti-mouse H-2Kd and analyzed by flow cytometry. Data were collected on a FACSCanto(Becton Dickinson) and analyzed using FlowJo software(Treestar)

12b

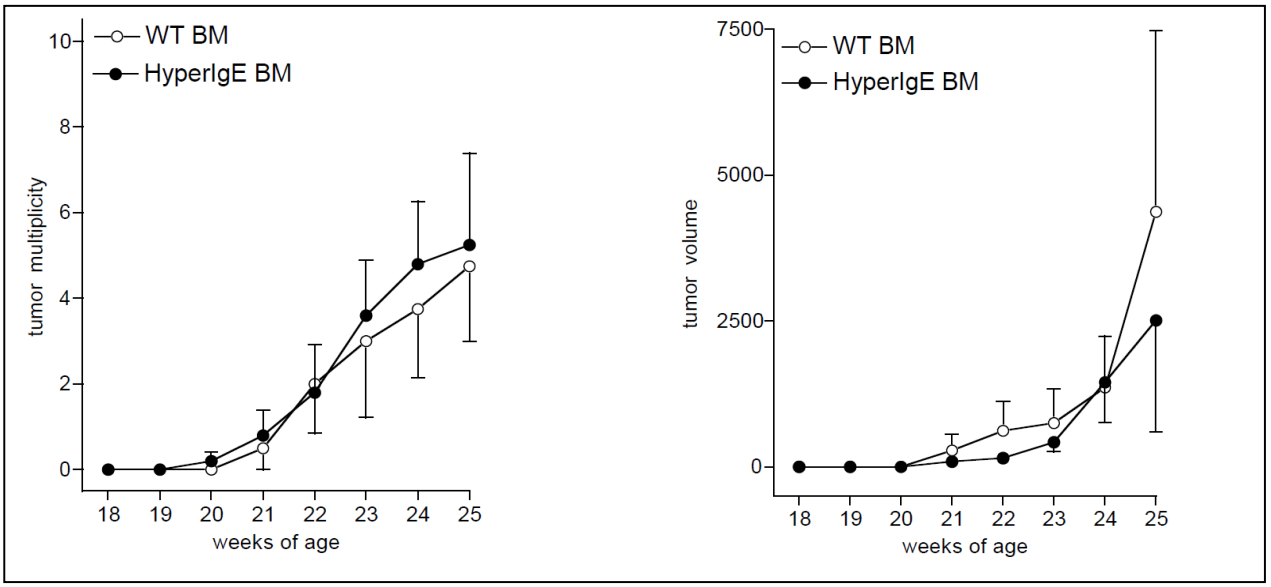


Figure 12b. Growth of Tumor in Her-2/neu BMT from HyperIgE and WT mice

12b.BMT from KN1-HyperIgE mice does not have any effect on suppression of tumor. There is no difference between BMT from WT and KN-HyperIgE mice group. Tumors were measured with a caliper once a week, and tumor volume is calculated as d2 × D, where d is the smaller diameter and D is the longer one(Values are in Mean±SEM).

3.3.2 Does BMT followed by DNA vaccination has any response in anti-tumor suppression

Previously it has been studied that DNA vaccination against Rat Her-2/neu p185 was effectively able to inhibit tumor growth(Stefano *et al.,* 2000).Since KN1-HyperIgE mice were able to induce anti-tumor protection with *in vivo* endogenous experiment but not the same effect when bone marrow cells are transplanted to spontaneously tumor developing mice, experiment was set up to assess if KN1-HyperIgE mice which are Bone Marrow transplanted and further boosted by vaccination are able to suppress tumor growth.

BMT was performed first from KN1-Hyper IgE mice to HER-2/neu mice at the age of 10 weeks. After 4 weeks of BMT, mice were vaccinated with plasmids coding for extra-cellular and transmembrane domain of the product of transforming rat Her-2/neu oncogene. Plasmids(50µg/injection) were injected into quadriceps muscle through 28-gauge needle syringe(Figure .13)

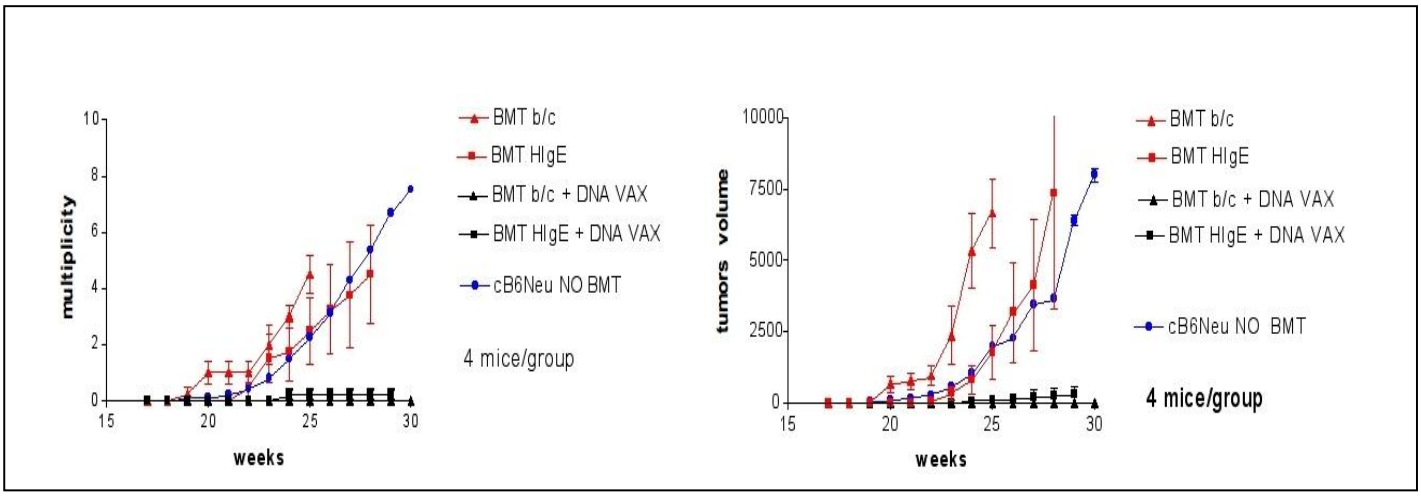


Figure .13 BMT followed by DNA vaccination able to bring down tumor growth

BMT mice from KN1-HyperIgE and wild type at 10 weeks of age followed by DNA vaccination at 14 week able suppress tumor growth.pCDNA3 coding for Her2/neu were injected into quadriceps muscle through a 28-guage needle syringe. Tumor suppression is seen even wild type group,is not specific to KN1-HyperIgE group.

There was no tumor growth suppression or delay observed when bone marrow cells are transplanted from KN1-HyperIgE mice. The endogenous IgE anti-tumor suppression seen *in vivo* experiments are not replicated in BMT experiment using the parental HER-2/neu mice from which N2C cells are derived. The tumor growth was seen in all the 10 glands (tumor multiplicity) and growth in tumor volume for BMT groups was consistent indicating no transfer of antitumor effect. In groups where BMT was followed by DNA vaccination, there was no tumor growth observed in

KN1-HyperIgE BMT group, but this protection was due to DNA vaccination as this effect of tumor protection was even seen in groups BMT from wild type.

3.4 Generation of new Transgenic model to show a direct role of IgE in anti-tumor activity

To demonstrate that IgE was the factor responsible for anti-tumor activity observed in previous *in vivo* studies and this mechanism was due to its interaction with its high affinity receptor FcεRI, experiments were set-up making use of FcεRIα *knock out* transgenic. It has been already shown in earlier studies (Nigro.*et al.,* 2009) the role of IgE-FcεRI interaction in tumor surveillance and suppression. FcεRIα *knock out* and KN1-HyperIge mice were inter-bred so as to arrive at a phenotypic condition wherein the mice will be high IgE producing but its high affinity receptor is knocked down, thereby IgE without its receptorFcεRI is functionally obsolete.

The breeding program to generate this transgenic was followed as per principles of Mendel. The breeding plan started with mating of male KN1-HyperIgE with female FcεRIα *knock out* transgenic mice and vice versa. The average size of the litter was 4pups/litter for F1 and it drastically increased to an average size of 7-8 pups/litter in F2 condition. All along the breeding program the mice were healthy, fertile and had normal reproductive cycle. They did not show any signs for loss of viability and fertility and no symptoms for any diseases.

The mice genotype was determined in each step by PCR. In KN1-HyperIgE mice, the mutation is in targeted knock-in of chromosome 12, where membrane exons of the ε heavy chain is replaced by those of the γ1 heavy chain by homologous recombination in embryonic stem cells (Gertrude Achatz-Straussberger *et al.*,2008).

In FcεRIα *knock out* ,α chain in receptor gene for high affinity IgE has been knocked off at exon 4 by addition of a neomycin cassette(Dombrowicz *et al.,* 1993) and hence cannot bind to IgE and loss of function is noted.

3.4.1Breeding Plan

Breeding between KN1-HyperIgE and FcεRI knock out (KO) transgenic

P= KN1-HyperIgE FcεRI KO

RRHH ⤫ rrhh

⇩

F1=

RrHh ⤫ Rr Hh

F2=

F2	**RH**	**rH**	**Rh**	**rh**
RH	RRHH	RrHH	RRHh	RrHh
rH	RrHH	**rrHH**	RrHh	rrHh
Rh	RRHh	RrHh	RRhh	Rrhh
rh	RrHh	rrHh	Rrhh	rrhh

Genotype		RRHH	RrHH	rrHH	RRHh	RrHh	rrHh	RRhh	Rrhh	rrhh
Ratio		1	2	1	2	4	2	1	2	1

1: 16 = desired genotype = rrHH

Figure 14. Breeding Plan for HyperIgE mice with FcεRI *knock out* (KO)mice.

All the F1 progenies were in heterozygous condition(RrHh).For F2 condition the segregation of alleles are as per Mendelian ratio. Therefore the desired mouse (high IgE producing but receptor knocked down) would be achieved in Mendelian ratio of 1:16 in F2. The F1 heterozygous mice were of much more vigour than their parents as the litter size increased from average of 4 to 8 pups/litter.

Segregation of alleles following Mendelian law

For n = 46

Genotype	RRHH	RrHH	rrHH	RRHh	RrHh	rrHh	RRhh	Rrhh	rrhh
Mendelian ratio	1:16	2:16	1:16	2:16	4:16	2:16	1:16	2:16	1:16
Expected ratio (n= 46)	3	6	3	6	12	6	3	6	3
Observed ratio(n= 46)	5	6	4	5	7	6	2	7	4

For n = 119

Genotype	RRHH	RrHH	rrHH	RRHh	RrHh	rrHh	RRhh	Rrhh	rrhh
Mendelian ratio	1:16	2:16	1:16	2:16	4:16	2:16	1:16	2:16	1:16
Expected ratio (n=119)	7	15	7	15	30	15	7	15	7
Observed ratio(n=119)	21	16	3	18	17	16	12	8	8

Figure .15 Initially to start with, Mendelian ratio for segregation held good, but as the mating samples were increased, Menedelian ratio was lost.

F2 Breeding Plan

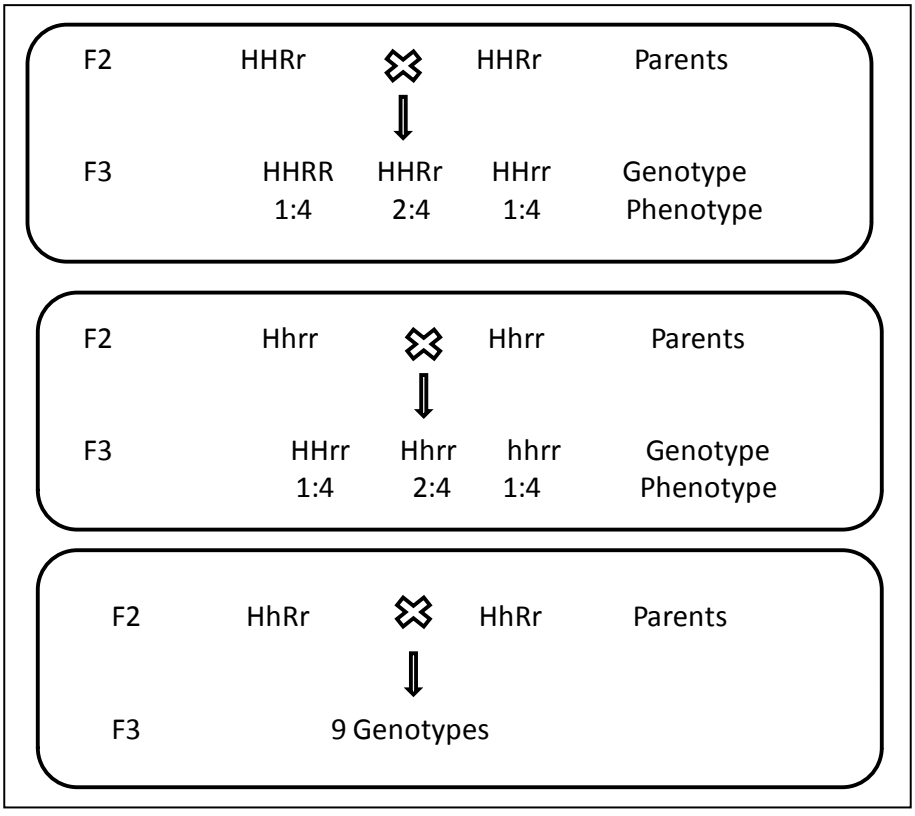
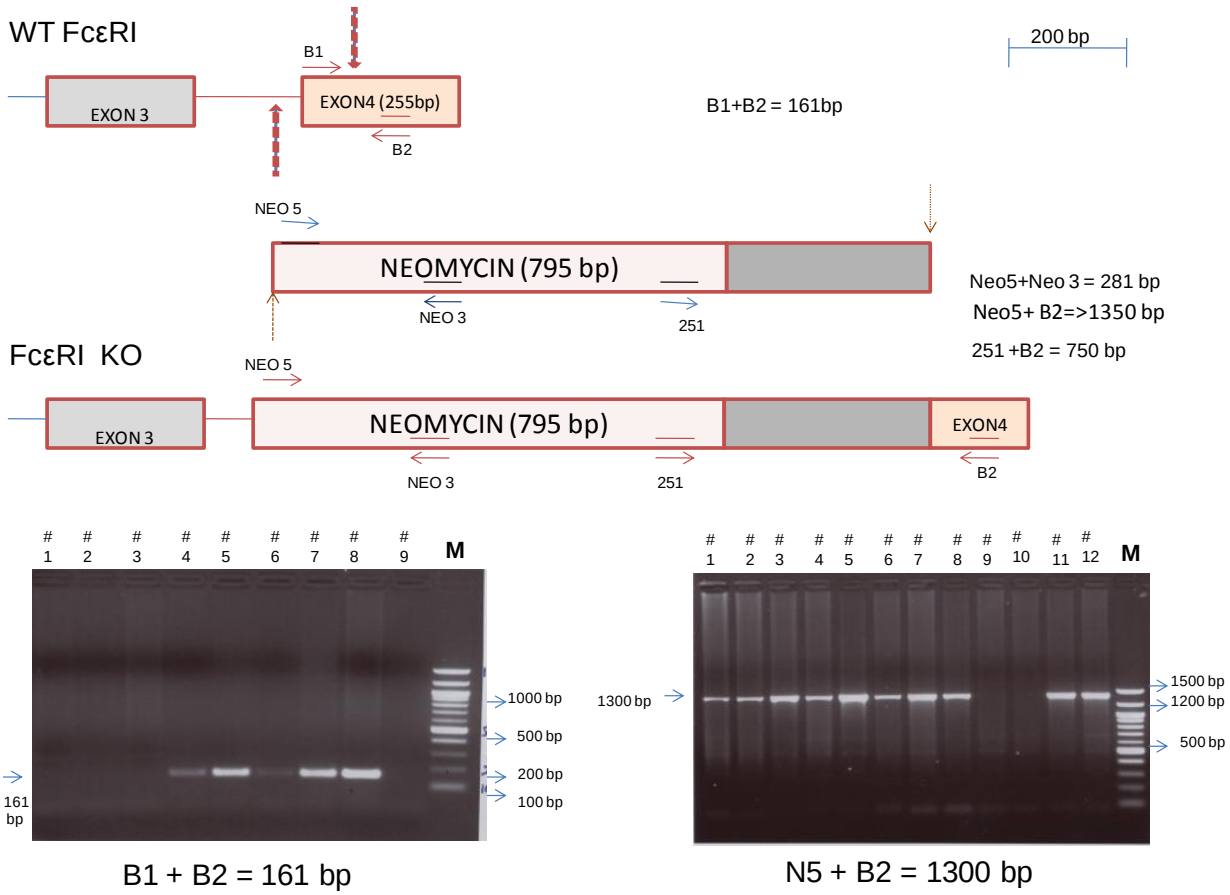


Figure .15b –In F2 Breeding to maximize the production of desired genotype (rr HH),the parents considered were such that minimum 25% of the progeny will be of desired mice. To maintain different genotypes, heterozygous pair was in breeding.

3.4.2 Genotype confirmation by PCR

For FcεRI gene

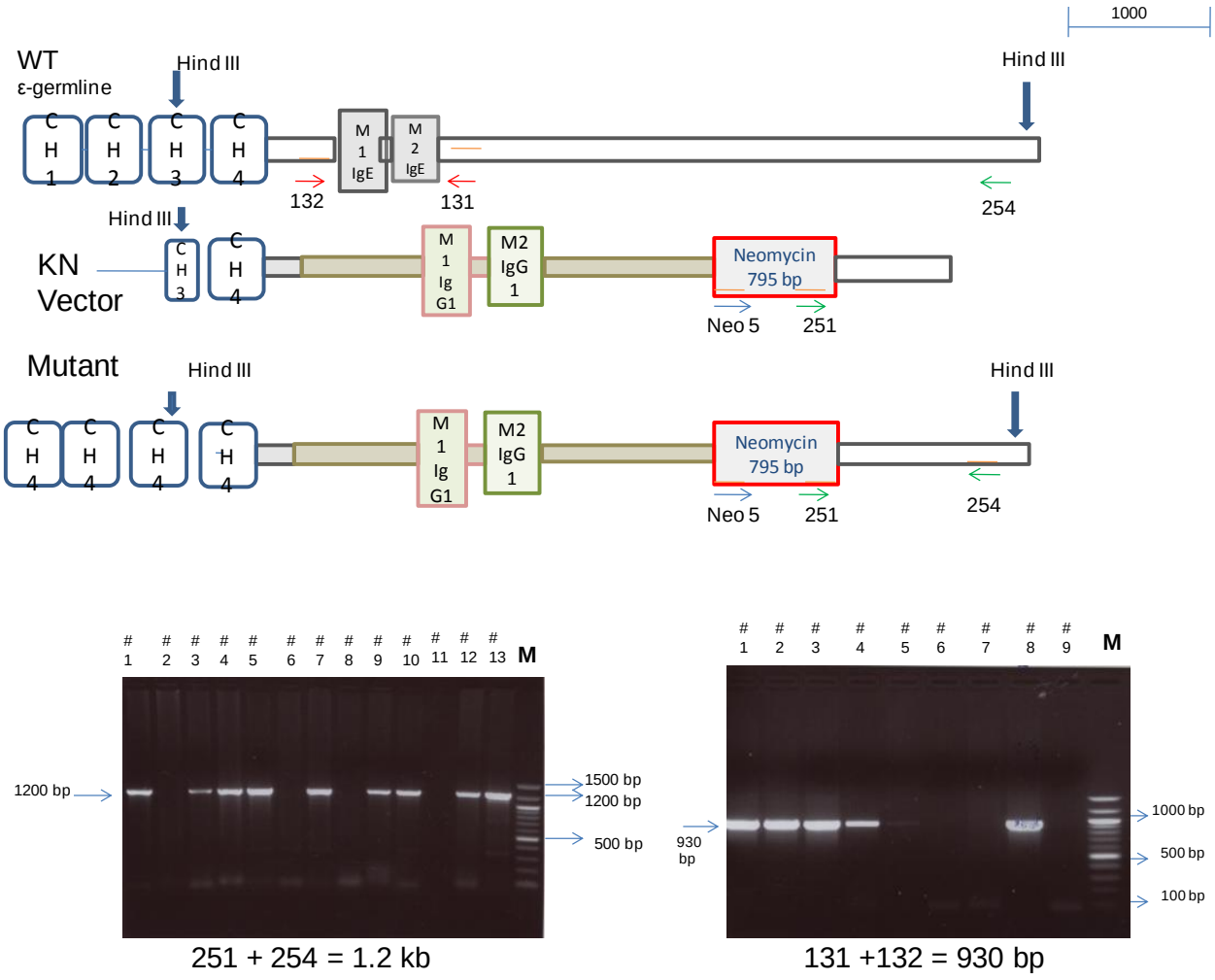


Gene	*Primer ID*	*Primer base Position*		*Sequence(5'-3')*	*Tm*
mFcERI α exon4(255 bp)	**B1**	2-21	*Forward*	TTg gCT gCT CC TTC AgA CAT	60 ®
mFcERI α exon4(255bp)	**B2**	141-161	*Reverse*	CTC TCT AAT ggA gAC ggggC	60 ®
Neomycin (795 bp)	**NEO '5**	41-60	*Forward*	CTT GGG TGG AGA GGc TAT TC	60 ®
Neomycin (795 bp)	**NEO '3**	301-320	*Reverse*	AGG TGA GAT GAC AGG AGA TC	60 ®
Neomycin (795 bp)	**251**	703-723	*Forward*	GAA TGG GCT GAC CGC TTC CTC	60 ®

Figure 16 – Genotype was confirmed by PCR analysis.

To detect and distinguish between wild type and FcεRIα KO allele primers were designed and PCR setup. The wild type allele amplified at 161bp and knock out was detected by using a neomycin primer and B2 exon primer which amplified at 1300 bp.

For IgE gene



251 + 254 = 1.2 kb

131 +132 = 930 bp

Gene	*Primer ID*	*Primer base Position*		*Sequence(5'-3')*	*Tm*
IgE germline (5667 bp)	**132**	273-301	*Forward*	GAA ATG GAC CTA TAA GCT TAG AGC CTT CC	60 ®
IgE germline (5667 bp)	**131**	1170-1195	*Reverse*	GAT GTT CTT CTA AGC TTT GTC TCA AAG	60 ®
IgE germline (5667 bp)	**254**	3733-3754	*Reverse*	GGT AAA GGC TGC TTC CTA GTC	60 ®
Neomycin (795 bp)	**251**	703-723	*Forward*	GAA TGG GCT GAC CGC TTC CTC	60 ®
Neomycin (795 bp)	**Neo '5**	41-60	*Forward*	CTT GGG TGG AGA GGc TAT TC	60 ®

Figure 17. PCR analysis for IgE gene

The presence of IgE gene and its distinction to KN1-HyperIgE allele was done using PCR. Wild type IgE amplified at 930 bp and HyperIgE allele was amplified at 1200bp.

3.4.3 Anti-tumor effect seen in Intermediate Genotypes.

When mice intermediate to the desired genotype are challenged with tumor cells, there is varied response to tumor suppression seen, in accordance to presence or absence of receptor FcεRI and IgE production. Mice of different genotype from the cross were challenged, the growth of tumor was monitored up to 30 days post tumor cells challenge or till tumor size exceeded above 500mm$^{3.}$ (Figure .18)Blood was collected prior to challenge, day 15 and 30 post tumor challenge and analyzed for specific IgE release.

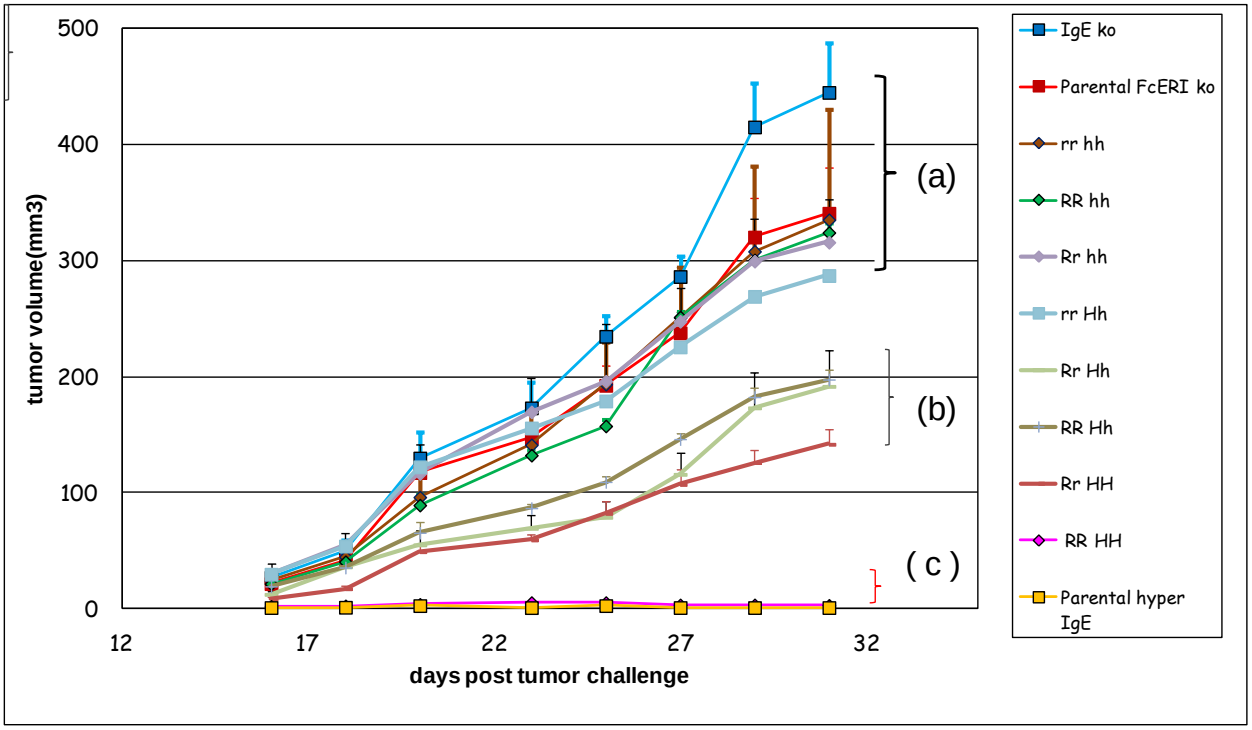


Figure 18. Mice from F2 cross showing varied response to tumor growth.

The tumor growth graph of the experiment point towards three distinct categories which we can place the genotypes into. In the first category (a), IgE KO(blue line), FcεRIα K0,parental (red line)and from the crossing(dark red), wild type genotype(green line),mice with genotypes where the receptor is lost(knocked off) and IgE allele is in wild type condition ,these group show highest tumor growth . The second category (b) there is considerable reduction in tumor growth, wherein the alleles for KN1-Hyper IgE and FcERI are in heterozygous condition. In the third category(c), the mice are in High IgE production with normal intact receptor FcεRI condition, are able to completely suppress the tumor growth.

The tumor growth showed a pattern which can be categorized into 3 divisons. The first divison (Fig.18a) comprised of mice groups of IgE *knock out*, FcεRIα *knock out*, mice with genotype rr hh (equivalent to parental FcεRI *knock out* mice,RR hh(wild type genotype),Rr hh,rr Hh. The tumor growth in all these groups was highest indicating the importance of receptor FcεRI and elevated

IgE levels in tumor protection. The second divison (Fig.18b) included mice from genotypic groups RrHh, RRHh and RrHH. Tumor growth in these was significantly smaller than the first division(a) indicating that with at least one copy of KN1 allele and one copy of wild type receptor FcεRI,tumor growth comes down . In the third division (Fig.18c) comprising of Parental KN1-HyperIgE mice and its equivalent from the cross(RRHH), tumor protection was seen. The results from the last group also signified that there was no losses of KN1-hyperIgE gene in the crossing over.The tumor free mice from these two groups were subsequently challenged and no tumor growth was observed.

3.4.3 *In-vitro* Characterization for production of N2C tumor specific IgE in mice determined by functionality assay

Serum collected for *in vivo* experiment was analyzed for release of specific IgE against tumor.The experiment was setup with previous conditions. The release of β-hexosaminidase by RBL-2H3 was detected in the supernatant. The 100% release positive control was obtained by stimulating RBL-2H3 with mIgE cross linked with soluble anti-IgE DNP -HSA,nonstimulated RBL-2H3 cells was used as control. Values are mean ±SD of three determinations.

There was no detection of specific IgE as determined by release of mediators. Only the groups of parental HyperIgE and RRHH- genotyped mice (phenotypically equivalent to KN1-HyperIgE) there was increase in mediator release and which was comparable, corresponding with *in vivo* data that there is no loss of HyperIgE gene in the breeding. It was also evident that on subsequent challenges there is increase in specific mediator release signifying establishment of IgE memory cells and increase in specific response upon re-challenge.

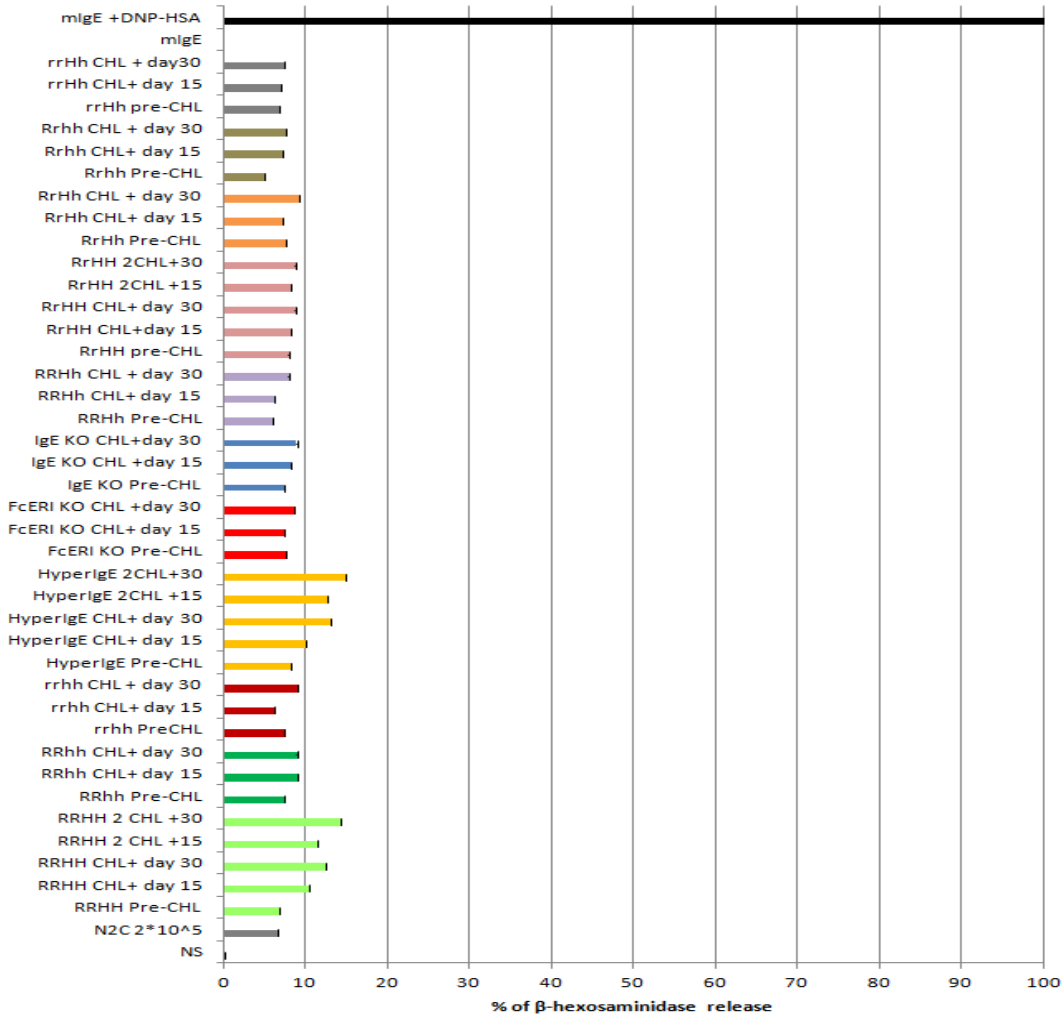


Figure 19- IgE mediated β-hexosaminidase release assay

There was no increase in specific IgE seen along different genotypes. Only in KN1-HyperIge mouse and RR HH genotype mouse group there was slight increase in release. Results are mean± SD of three determinations.

3.4.4 Induction of role of IgE through its interaction with high affinity receptor for anti-tumor activity.

Experiment was set-up to demonstrate the role of IgE through its high affinity receptor FcεRI for its anti-tumor potency as seen in previous studies (chapter 3.2). From the breeding program Transgenic mice which is double mutant (rr HH), having the function of high IgE production but with its FcεRI receptor knocked off were used along with KN1-Hyper IgE, FcεRI KO and wild type mice. N2C cells were challenged on the left flank (6×10^5 cells in 0.2ml of PBS).Subsequently the growth of tumor was monitored up to 30 days post tumor cells challenge or till tumor size exceeded above $500mm^{3.}$ (Figure .20)Blood was collected prior to challenge, day 15 and 30 post tumor challenge and analyzed for specific IgE release.

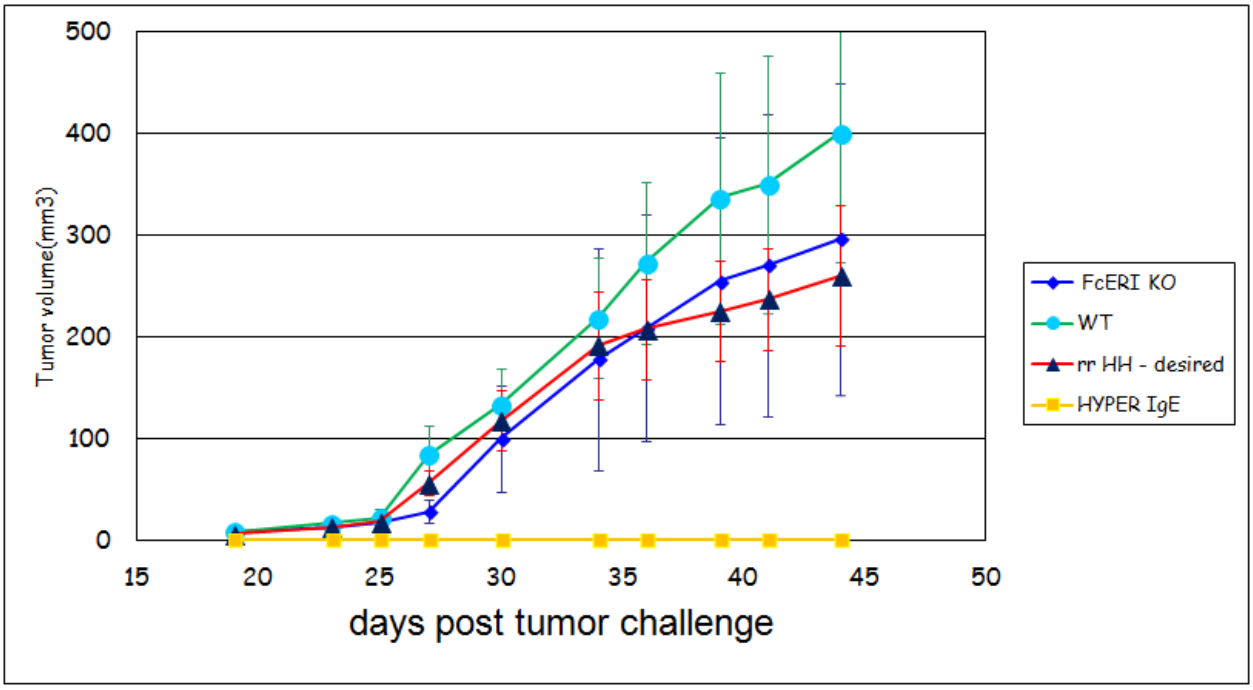


Figure 20- Anti-tumor protection seen is due to IgE and protection is acquired through interaction with its high affinity receptor FcεRI

IgE has a role in in induction of anti-tumor property and this potency for Tumor protection is through its interaction with its receptor FcεRI.The double mutant mice has similar high production of IgE to KN1-HyperIgE mice but lack in FcεRI receptor. Whereas tumor protection is seen in KN1-Hyper IgE group protection is lost in the newly generated double mutant clearly indicating role of IgE in observed tumor protection. Tumor develops in FcεRI KO and wild type group (Values are in Mean±SEM).

Analyzing the tumor growth , there was tumor protection seen as in HyperIgE mice, and growth of tumor in wild type and FcεRI KO mice and interestingly there was growth of tumor in double mutant (rr HH) group. This double mutant shared the same gene for high IgE production with KN1-HyperIgE, therefore having elevated IgE levels but differed by lacking in functional high affinity receptor gene. Herby the loss of tumor protection is due to absence of high affinity receptor FcεRI. This result clearly inducted the role of FcεRI receptor in IgE mediated immune response. The tumor free mice of HyperIgE group were subsequently challenged but no tumor growth was seen indicating again towards establishment of IgE memory cells against tumor.

3.4.5 Detection of specific IgE against tumor

In-vitro release assay was performed with serum samples obtained before to and after the tumor challenge and analyzed for specific IgE produced. Experiment was set-up as before with an additional step of including serum samples which were depleted of IgE by incubating with ant-IgE antibodies and assay performed like previously. The amount of release detected in supernatant will correspond with specific IgE against the tumor. The 100% release positive control was obtained by stimulating RBL-2H3 with mIgE cross linked with soluble anti-IgE DNP -HSA, nonstimulated RBL-2H3 cells was used as control. Values are mean ±SD of three determinations.

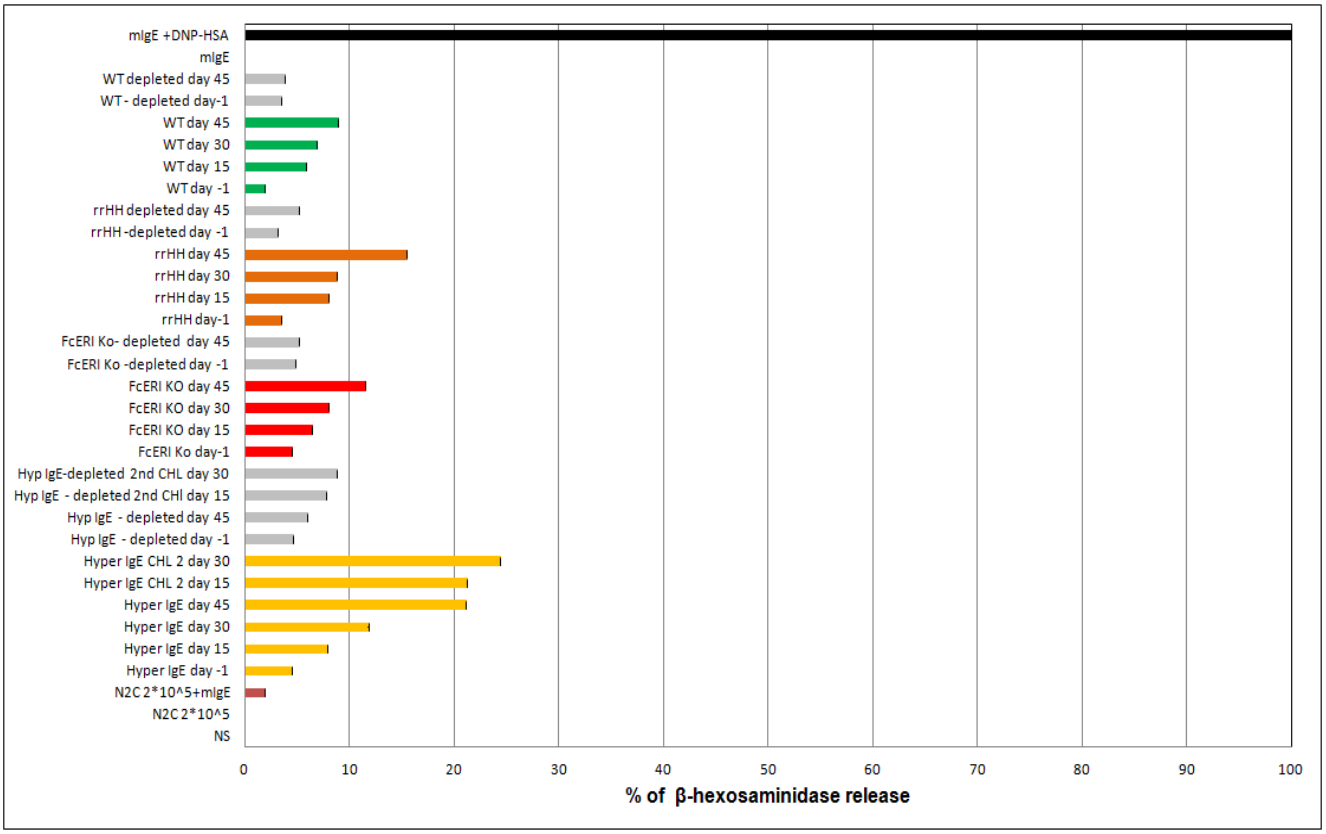


Figure .21 Specfic IgE against the tumor detected in serum samples .

IgE specific against N2C tumor cell by the way of mediator release is detected. The serum samples when depleted of IgE was not able to induce release of mediators or the release was of basal level comparable to wild type and FcERI knock out(ko) levels indicating that IgE is increased and production of tumor specific IgE upon exposure . (Values are in Mean±SD)

An increased specific IgE release against the tumor by way of mediator release was observed in serum samples of KN1-HyperIgE group which was not detected in wild type and FcεRIα KO groups. Interestingly, the double mutant group showed increased detection of specific IgE signifying that

there is induction of specific IgE against the tumor. To verify the release obtained is only due to specific IgE bound to tumor cells and not due to background of endogenous elevated IgE levels, serum samples were depleted of IgE using anti-IgE antibodies .The results show that there is no marked difference in mediator release for wild type and FcεRIα KO group but substantially reduced in serum samples collected on day 30 and 45 of KN1-HyperIgE and double mutant group. Even the serum samples after 2nd challenge with Tumor of KN1-HyperIgE group depleted of IgE showed drastic reduction in mediator release, indicating that release seen was due to specific IgE.

Also by the release results, it became evident that in Double mutant mice which have elevated IgE levels, there is production of specific IgE against the tumor but due to lack of functional high affinity receptor FcεRI ,IgE can't induct a antitumor response as seen in KN1-HyperIgE mice.

Ongoing Studies :

Effect of $CD8^+$ T cell depletion on efficacy in tumor protection

As in studies with exogenous IgE experiments(Reali *et al.*,2001), which had shown that by depleting CD8+ cells the antitumor effect was lost indicating the role of CD8 T-Lymphocytes in tumor immunity. CD8+ T cells play an important role in inhibiting and killing tumor cells and impeding tumor growth. CD8+ cytotoxic T lymphocytes (CTLs) destroy tumor cells as a result of triggering apoptosis, therefore they may have an effect on tumor growth. To investigate if $CD8^+$ lymphocytes were involved in acquiring of tumor protection, we depleted $CD8^+$ T cells with anti-CD8 ascites. The mice were injected intravenously with 300µg of ascites at day -7 and 200ug on day -4and -2 days. This boost challenge of anti-CD8 was done to completely eliminate the CD8+ cells.

KN1-HyperIgE mice were considered for depletion studies as they were able to suppress growth of Tumor. As control KN1-HyperIgE mice without depletion, wild type and FcεRIα KO, double mutant (rr HH) and Rr HH(Hyper IgE with heterozygous for receptor was also included. Mice were challenged with N2C cells(6×10^5 in 0.2ml PBS) s.c on left flank. Tumor growth was monitored for upto day 31.(Figure) Periodically blood was collected to check for depletion by Immunofluorescence. CD8 depletion was seen >90% compared to controls. Mice were boosted again after 2 weeks and 3 weeks after challenge to maintain this depletion ratio.

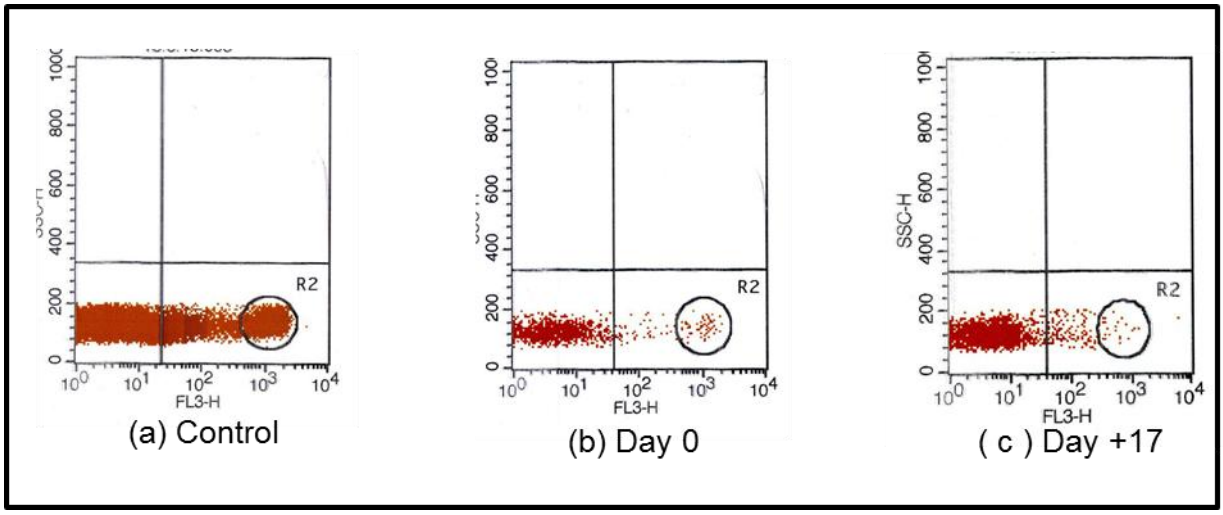


Figure .22 FACs analysis to confirm $CD8^+$ depletion- Blood collected from KN1 mouse was analyzed for $CD8^+$ depletion. Compared to undepleted KN1-HyperIgE mice(a),depletion was >90% on day 0 (b), and > 95% on day 17 post tumor challenge.

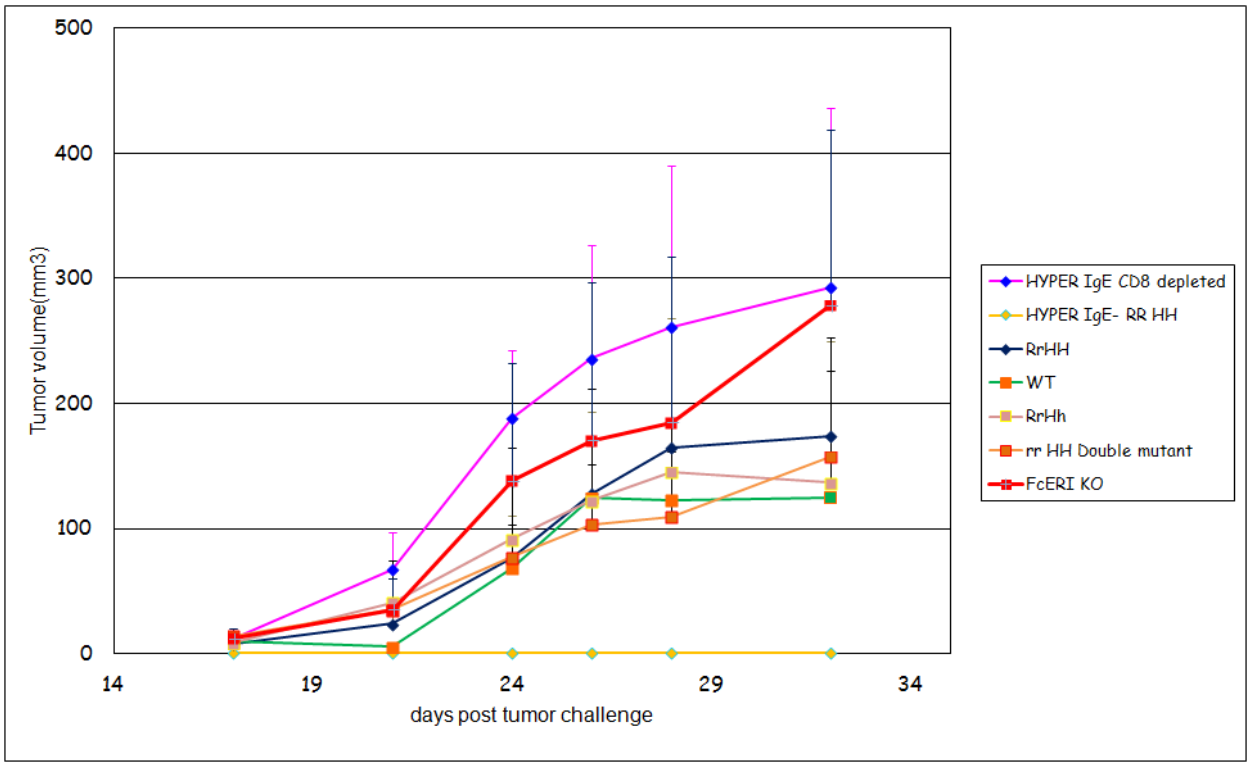


Figure .23 Depletion of $CD8^+$ T cells enhanced tumor growth in HyperIgE mouse

KN1-HyperIgE mice depleted of $CD8^+$ cells showed growth of Tumor in comparision to undepleted KN1-HyperIgE group which had abolished tumor growth, thereby implicating role of $CD8^+$ T-lymphocytes in acquiring Tumor immunity. In the other groups, FcεRI knock out(KO),double mutant,wild type,and heterozygous group tumor growth is observed.

The tumor graph clearly inducted the role of CD8+ T-lymphocytes in tumor immunity. KN1(HyperIgE) mice group was able to suppress tumor growth completely, whereas in the CD8+ depleted KN1(HyperIgE) group the tumor protection was lost and the tumor growth observed was highest among the groups. As before, the *wild type,* FcεRIα KO, RrHh-(heterozygous for both genes), RrHH(heterozygous for receptor and Homozygous for HyperIgE gene) and rrHH- Double mutant(HyperIgE and lacking in receptor) groups the tumor growth was seen. The growth pattern of depleted CD8+ HyperIgE group was similar to that of FcεRIα *knock out* group

DISCCUSSION

Several studies report over the past two decades, the potential of Immunoglobulin E to induce tumor cell death(Nagy E *et al.,* 1991;Kershaw MH *et al.,*1998;Gould HJ *et al.,*1999; Reali E *et al*., 2001;Riemer AB *et al.,* 2007).Previous pioneering *in vitro* and *in vivo* studies have revealed that engineered anti-cancer IgE antibodies may be comparable or even superior to their IgG counterparts (Gould *et al.,* 1999, Karagiannis *et al.,* 2003, Karagiannis *et al.,* 2007, Karagiannis *et al.,* 2008, Karagiannis *et al.,* 2009). These studies were based on epidemiological data that that widely discussed in connection with a possible inverse correlation between the presence of allergic disorders and the incidence of tumors (MC Turner *et al.,* 2006). According to these studies high serum levels of IgE might contribute to the prevention of tumor development.

Although many therapeutic mAbs exert their anti-tumour effects through a multitude of mechanisms such as Fc-mediated mechanism of immune system engagement that play a key and important role (Woof, 2012, Moore *et al.,* 2010). Recent data support the substantial contributions of Fc-mediated mechanisms clinical efficacy of therapeutic antibodies. Indeed Fc-mediated mechanisms of action are attributed to the efficacy of antibodies, such as trastuzumab in the clinical use (Nahta and Esteva, 2007, Karagiannis *et al.,* 2009, Lazar *et al.,* 2006), cetuximab (Jonker *et al.,* 2007) and the recently approved ipilimumab (Lipson and Drake, 2011). These antibodies lend credit to the notion that effector cell activation is a crucial contributor to the antitumoral properties of therapeutic mAbs.

Interaction of a therapeutic antibody with immune effector cells is mediated by its Fc domain, which determines not only its binding to complement, but also its binding to the relevant cellular Fc receptors Thus, it is clear that the class or subclass of an antibody critically determines its effector functions, and ultimately could influence efficacy. Therefore, one strategy to optimize the antibody-immune system interaction exploration of the use of antibodies with Fc regions of alternate Ig classes, such as those of the IgE

Indeed, work in this area constitutes an important branch of the rapidly growing field of Allergo-oncology that aims to address the potential opportunities of IgE-mediated and Th2-biased cellular responses in malignant diseases (Jensen-Jarolim *et al.,* 2008).

Manifestations of allergic disease and immune surveillance in parasitic infections, namely local immune stimulation, with an ensuing cascade of ‘allergic’ inflammation at the site of antigen provocation, could be harnessed to re-direct the potent immune cell populations to induce tumor

rejection. The potential advantages of IgE antibodies in the treatment of solid tumors(reviewed in chapter 1)) could motivate the research groups to develop not only tumor-specific recombinant IgE antibodies,but also other immunotherapeutic approaches aimed at triggering IgE expression for targeting tumor cells (Nagy *et al.,* 1991, Daniels *et al.,* 2012, Teo *et al.,* 2012, Karagiannis *et al.,* 2012, Kershaw *et al.,* 1996, Kershaw *et al.,* 1998).

Previous studies in our laboratory have revealed the ability of mouse IgE on tumor growth suppression and antitumor immunological memory in C57BL/6 mice (Reali *et al.,* 2001). In that study, IgE tumor cell loading was obtained by a three-step strategy, based on a biotin-avidin bridge. The vaccination protocol immunizations with IgE- or IgG-loaded irradiated tumor cells, followed by a challenge with live tumor cells. IgG loading did not show any statistically significant difference in tumor protection. Conversely, mice vaccinated with IgE-loaded cells showed a powerful tumor protection, indicating that IgE can exert an adjuvant effect. The involvement of eosinophils, CD4+T cells and CD8+T cells were appeared to be crucial because the depletion of any of these cell types abrogated tumor protection.

Moving further ahead, studies have investigated the IgE antitumor adjuvanticity, by probing the involvement of FcεRI. It could be analyzed that the IgE adjuvanticity observed in the antitumor vaccination would have resulted from an inflammatory reaction, similar to those induced by IgE in allergic manifestations. Because such reactions are mediated by FcεRI activation and studies were performed to investigate the dependence of the IgE antitumor effect upon FcεRI activation. To this objective, the availability of BALB/c FcεRI -/- and CD23-/- mice was exploited. First, the IgE protective effect was confirmed in a BALB/c tumor model, where IgE-loaded MVA-infected tumor cells proved to be an effective cellular vaccine. Second, FcεRI -/-mice vaccinated with IgE- loaded MVA-infected tumor cells did not show any antitumor protection, demonstrating that FcεRI plays a crucial role in *in vivo* in IgE adjuvanticity. Third, CD23-/- mice vaccinated with IgE-loaded MVA-infected tumor cells showed antitumor protection indicating that CD23 is not involved in the IgE adjuvant effect, thus reinforcing the unique importance of FcεRI. The results reported in these studies strongly suggested that the IgE adjuvant effect in antitumor vaccination is mediated by the IgE-FcεRI interaction through contact between IgE loaded tumor cells and FcεRI(Nigro *et al.,*2009)

Epidemiological studies have suggested the inverse associations between allergic diseases and malignancies, and atopic patients are characterized by high IgE levels in serum. Though presence of high serum levels of IgE contributes to the prevention of tumor development, an

endogenous IgE with TAA specificity derived from cancer patients or healthy individuals is still need to be discovered. However, initial efforts in this direction are being made.

A combination of knowledge in active cancer immunotherapy on the one hand, and basic allergy mechanisms on the other, prompted the development of a vaccine that would induce tumor-specific IgE in *in vivo*. Two strategies were combined: first, an epitope-specific vaccination against the tumor antigen HER-2, rendering antibodies with similar biological properties as that of the monoclonal antibody trastuzumab (Knittelfelder *et al.,* 2009)Second, an oral immunization regimen, discovered in food allergy research, that might resulting in IgE induction (Riemer *et al.,* 2007). However, IgE antibodies favor the recognition of conformationally intact antigens with a dense epitope display. Intact, viable tumor cells fulfill these requirements because they overexpress antigens via interaction with its receptors on numerous defence cells. Conversely, IgE directs potent effector cells into tumor tissues with proven tumoricidic activity. Thus it can be hypothesized that IgE antibodies might physiologically survey the malignant cells. Based on the above data and previous findings in our laboratory on role of exogenous IgE in tumor adjunctivity, together with the numerous and controversial epidemiological studies regarding a possible link between allergies and anti-tumor (MC Turner *et al.,* 2006), we decided to carry out detailed investigation on a possible role played by intrinsic IgE in antitumor potency developing three lines of study

Firstly, we investigated if host IgE(endogenous) is able to induce an immune response upon challenge against tumor cells as IgE effect involved in previous antitumor experiments was due to exogenously loaded IgE (Nigro *et al.,*2009).

For the initial studies, presence of different transgenic mice models, engineered high IgE producing (Hyper IgE), IgE *knock out*(KO)(Achatz G *et al.,1997*) along with wild type mice were considered to investigate the possible involvement of endogenous IgE in immunity against tumors. Physiologically, IgE production in serum is increased in KN1-HyperIgE mice compared to wild type and negligible in knock out condition.

Studies were started using the cell line TS /A-LACK, mammary adenocarcinoma (Benigni F *et al.,* 2005). Experimental results clearly indicated that in the case of KN1-Hyper IgE mice group with one immunization the tumor growth was abolished, whereas in group without the immunization the tumor growth was significantly less than groups of wild type and IgE KO with or without immunization. Also the results clearly indicated the role played by IgE in tumor protection as in the case of IgE *knock out* mice there was loss of tumor protection. The tumor growth in the

wild type group but absence in the KN1-Hyper IgE group indicates that elevated levels of IgE are required to have antitumor potency. Upon subsequent challenge of tumor cells on tumor free KN1-Hyper IgE mice, there was no tumor growth observed. These results indicate that IgE antibody may have role in the production of Tumor specific memory cells for enhancing antigen presentation thereby stimulating a secondary T-cell-mediated response in turn that would enhance the *in vivo* anti-tumor immune response for subsequent challenges. *in vitro* IgE mediated hexosaminidase release assay was performed for evaluating the production of specific antibody against the tumor. There was no detection of increase in mediator release among the groups in contrast to the group in which KN1-HyperIgE mice has been subsequently challenged twice, a significant increase of mediator release was observed indicating presence of specific tumor specific IgE antibodies in serum.

Vaccinating with irradiated tumor cells, we allowed the host immune system to sensitize and react against tumor antigens.

We had assumed, if there is anti-tumor intrinsic power of IgE, following inoculation of live tumor cells in the three mouse strains previously vaccinated, then the antitumor effect will be highest in KN1-HyperIgE mice, less prominent in wild type and completely absent in IgE knock-out. Surprisingly the data revealed a full antitumor protection after immunization in HyperIgE mice and comparable tumor growth in other two strains of mice. It was first evidence that the endogenous IgE play a role in immunity against tumors

Subsequent studies were conducted in a different tumor model to minimize model bias. N2C tumor cell was considered since, it over expresses HER-2/neu antigen on its surface and hence can enhance the desired anti-tumor effect. Initially to fix the minimal tumor cell count required for 100% tumor growth in different in IgE KO and KN1-Hyper IgE, experiment was set-up without immunization. Interestingly we observed that even without immunization KN1-HyperIgE group was able to completely suppress tumor growth; in contrast tumor growth was observed in IgE KO groups. For this study we included a CD-23 KO transgenic, to evaluate its role in antitumor effect as we had seen from previous studies with exogenous IgE(Nigro *et al.,*2009) that it had no role in tumor protection. Upon subsequent N2C tumor challenges in mice of this group no tumor growth was observed indicating the presence of IgE-memory cells against the tumor. In IgE-mediated release assay, there was significant increase in mediator release as compared to other group. Also the release increased in KN1-HyperIgE groups with subsequent challenges pointing towards induction of IgE specific memory cells. These results clearly indicated towards intrinsic

IgE's power in anti-tumor effect. The results also confirmed that CD23 does not have a role in establishment of tumor protection and suggested towards the prominence of receptor FcεRI in antitumor response.

Further to test this power of endogenous IgE of Hyper IgE mice to elicit an antitumor response Bone Marrow studies was set up in collaboration with the laboratory of Dr.M.P.Colombo in Tumori Institute, Milan. HER-2/neuT transgenic mice, which spontaneously develops tumor by 10 weeks of age and dies by 33 weeks of age was considered. The N2C tumor cell line used in previous study was derived from these HER-2/neuT mice and since Hyper IgE mice were able to suppress tumor completely even without immunization. BMT was performed taking cells from femur cells from HyperIgE mice and injecting into immunocompromised HER-2/neuT mice.We were not able to see any tumor suppression in KN1-HyperIgE BMT HER-2/neuT mice. Further, study was performed to check if boosting with DNA vaccination with plasmids encoding transmembrane domains of HER-2/neu after BMT can suppress tumor. Although there was no tumor growth, this effect was seen in control wild type indicating that the anti-tumor response was due to vaccination and not of Endogenous IgE response. One of the reasons for failure can be attributed to the fact that HER-2/neu mice starts to develop atypical hyperplasia by 7 weeks and by 11 weeks in situ carcinoma as in studies performed (Ambrosino *et al.,* 2006). This might be one of the reason as BMT performed at 10 weeks might be too late to for IgE to have a desired ant-tumor effect.

Further we wanted to validate if indeed IgE is behind the antitumor response and probe the role of receptor FcεRI in this IgE mediated antitumor effect. A new transgenic mice was generated in animal facility of the department. Making use of available transgenic in the facility, FcεRIα KO was bred with KN1-HyperIgE mice.This new breed of transgenic mice has capacity to produce high IgE but lacks FcεRI receptor, thereby indirectly demonstrating that IgE is involved in tumor protection and also establishes the role played by IgE-FcεRI in antitumor response. Mendels breeding program was followed and the desired double mutant was achieved in F2 generation in the ratio of 1:16 When experiment was setup using these double mutants, mice lost tumor protection whereas KN1-Hyper IgE mice were able to abolish tumor growth. Tumor was observed in the control groups of wild type and FcεRI α KO. This clearly demonstrated that the anti tumor potency is due to IgE and this is mediated through interaction with its high affinity receptor FcεRI. *In vitro* assay was conducted to detect tumor specific IgE in the serum, the release for KN1-HyperIgE group was significantly more, as compared to wild type and FcεRIα KO groups.

Interestingly the release was also higher in desired double mutant indicating that there is production of specific IgE against tumor and due to the absence of high affinity receptor FcεRI, tumor protection is lost. This clearly correlated with the *in vivo* result.

When the 8 different genotypes from the breeding plan was challenged to determine their role in tumor response, it was evident that on presence of high affinity receptor FcεRI and high IgE production alleles in homologous condition ,then there is tumor suppression. Whereas receptor in homologous recessive condition, even if IgE production is high the tumor growth was observed. In heterozygous condition for both the genes tumor growth is in median level. The tumor growth is significantly reduced in the group which has alleles intact for High IgE and only one receptor for FcεRI indicating that receptor levels are increased upon elevated levels of IgE and has a significant effect on tumor growth.

These studies clearly inducted the role of IgE towards antitumor response and this effect is brought upon by IgE-FcεRI interaction.

Ongoing work

We are now investigating the role of CD8+ T cells in inhibiting and killing tumor cells and impeding tumor growth. Preliminary studies of KN1-HyperIgE mice with CD8 depletion shows that the tumor protection is lost indicating the role of CD8+ cells in tumor immunity. KN1-HyperIgE mice group was able to suppress tumor growth completely, whereas in the CD8+ depleted KN1-HyperIgE group the tumor protection was lost and the tumor growth observed was highest among the groups.

This thesis work makes a contribution to the evolving role of IgE mechanisms in the context of cancer. These findings contribute new knowledge to the emerging field of Allergo-Oncology (Jensen-Jarolim *et al.,* 2008, Jensen-Jarolim and Pawelec, 2012, Jensen-Jarolim and Singer, 2011).

Acknowledgments

It is with immense gratitude for lot of support, encouragement and help of my guide Prof. Anna.T. Brini whose patience, enthusiasm and motivation has been infinite and inspirational. I could not have imagined having a better advisor and mentor for my PhD study.

I am also indebted to my supervisors of this project Prof. Antonio Siccardi and Prof. Luca Vangelista, at Universita San Raffaele for their kind support, guidance and advice.

I also thank Prof. Alberto Panerai for continuous support throughout my PhD study.

I could not have done this work without Elisa Nigro who has been incredibly supportive and insightful mentor throughout. It was really special working with you and thank you for all the support and motivation.

I, thank all my friends and colleagues Laura de Girolamo, Elena Arrigoni, Deborah Stanco, Stefania Niada, Maria Ferreira, Silvia Molinari, Patrizio Mancuso for their kind support and encouragement throughout my PhD. I would also remember the kind support, encouragement and help from Elisa Soprana, Maddalena Panigada, Francesco Gubinelli, Francesca Bosè, Valentina Bianchi, Andrea Barbieri, Andrea Mannucci, Valentina Bernasconi, Marta Recagni, Helios Recalde, Andrea Orsi. You all guys made me lot comfortable and was joy to work with you. I will be missing you all a lot.

I also express my thanks and happiness to Dr M.P Colombo, Silvia Piconese and Claudia Chidoni in Tumori Institute,Milan for guiding and encouraging me in the studies collaborated.

I would also like to thank all the staff in Department for being supportive and helpful. I wish to express my gratitude to Dr Giuseppe Rossoni, Dr Silvio Trivulzio , Bruno Vicerè and Fabrizio Viscardi for guiding me in the animal facilities of Via Vanvitelli 32.

This thesis would not have been possible without the support of the Fondazione Anna Villa Rusconi,Varese who provided funding for the last six months of my PhD, I'm in deep gratitude for their kind economic support.

Lastly, but importantly I can't forget the love, sacrifice and blessings of my Family. I dedicate this thesis work to them.

I also thank University of Milan and Department of Medical Pharmacology, for giving me an opportunity to come to Italy, providing a platform to gain knowledge and get exposed to the beautiful country and culture. Forza Italia !

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