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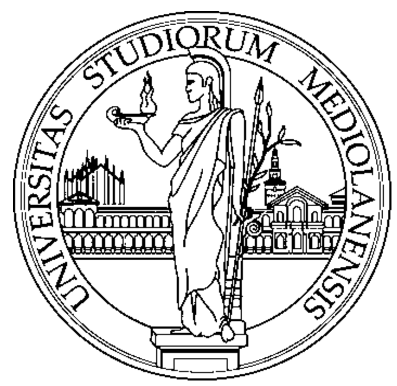
Department of Medical Biotechnology and Translational Medicine



DOCTORAL SCHOOL IN EXPERIMENTAL MEDICINE AND MEDICAL BIOTECHNOLOGIES

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Role of IgE in immunosurveillance: mechanism and potential therapeutic applications

Tutor: Prof. Diego Maria Michele FORNASARI

Supervisor: Dr. Anna Teresa BRINI

Head of PhD program: Prof. Massimo LOCATI

Lorena Maria Josè FERREIRA ESPINOZA

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"If you want to see a rainbow you have to learn to see the rain"

(Paulo Coelho)

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ABSTRACT

IgE is the class of immunoglobulins responsible for the protection against intestinal parasites and exerts a key role in the pathophysiology of allergic reactions. In addition, it has been demonstrated its involvement in the immune response against tumors in various animal models. According to our model, the function of IgE in the anti-tumor response is mediated by the binding of IgE with its high affinity receptor FcεRI expressed, in mice, on the surface of mast cells and basophils. Activation of FcεRI receptor leads to cell degranulation with release of preformed and newly synthesized mediators able to recruit effector cells that induce the establishment of a powerful inflammation at the tumor site. This inflammation, leading to cell death, could determine the processing of tumor antigens and the resulting immune response against tumor.

Based on promising data obtained previously in our laboratories, regarding the adjuvant effect of exogenous IgE in the anti-tumor vaccination and on the many controversial epidemiological studies about a possible link between allergies and cancer protection, we decided to investigate the possible role of endogenous IgE in the immunosurveillance of tumor. The use of two transgenic mouse models, one *knock-out* for the production of IgE (IgE-KO mice) and the other high IgE producer (KN1 mice), allowed us to investigate the possible involvement of host endogenous IgE in the immunity against cancer. Either in the absence or with a normal amount of IgE, tumor growth, preceded by vaccination with irradiated TS/A tumor cells (mammary adenocarcinoma), is not hindered and is comparable to not immunized mice. Differently, in high IgE producer mice, a single immunization was sufficient to obtain a complete anti-tumor protection. Moreover, challenging mice with a different tumor cell line, N2C tumor cells (less aggressive than TS/A tumor cells), the anti-tumor protection is observed even without immunization in 100% of KN1 mice compared to IgE-KO and wild type control mice.

To demonstrate that the protection observed in KN1 mice is due to the interaction of IgE with its high affinity FcεRI receptor, we decided to delete the FcεRI alpha gene in KN1 mice to remove their receptor. For this purpose we crossed KN1 mice with FcεRIα-KO mice, in order to obtain a double-mutant mouse model (DM), characterized by elevated levels of IgE but lacking in the expression of the high affinity receptor for IgE. The inoculation of N2C tumor cells in DM mice showed that the previously anti-tumor protection, observed in KN1 mice, has been widely lost and this is the fundamental point of this study because demonstrates that IgE-FcεRI axis is the basis of the role of IgE in anti-tumor immune response.

We also demonstrated, through an *in vitro* test of mediators release, the existence of tumor-specific IgE in the serum of KN1 and DM mice that were challenged *in vivo* with N2C tumor cells; moreover, the depletion of IgE from sera of KN1 and DM mice validate the specific contribution played by IgE in the mediators release.

Another point of this study was to understand how IgE acts in the observed anti-tumor effect. KN1 mice depleted of CD8+ T cells prior to tumor cells inoculation lost the anti-tumor protection. This result allowed us to point out that the anti-tumor immunosurveillance, driven by IgE, is largely mediated by an immune response dependent on CD8+ T cells. Next, we decided to isolate tumor-specific IgE through hybridoma technology, starting from spleen or bone marrow of KN1 mice that previously rejected the tumor *in vivo*. We tried to isolate tumor-specific IgE to employ them either in preventive vaccination or in therapeutic treatment protocols, in mice with already developed tumors, but unfortunately we were able to isolate a very low number of IgE secreting hybridoma that didn't show any specificity against the tumor cells.

Without any tumor-specific IgE, we planned a different approach for treating solid tumors, through intratumoral injection of rMVA-tmIgE (Modified Vaccinia Virus Ankara expressing a truncated form of the human membrane IgE), previously developed in our laboratories. This rMVA-tmIgE allowed to obtain tumor-infected cells expressing the Cε3Cε4 fragment of human IgE on their cell surface. Since MVA does not replicate among the infected cells without any effect on the surrounding host cells, the expression of Cε3Cε4 is restricted only in the tumor.

Several studies have also demonstrated the efficacy of the intratumoral treatment (i.t.) with vectors based on MVA, showing a consequent reduction of the tumor mass volume and an increased survival of treated mice. For this reason, in the last part of this project, we have evaluated the ability of rMVA-tmIgE to express, *in vivo*, the human membrane IgE on the tumor cells surface after intratumoral treatment of wild type mice with the vaccine. N2C tumor mass, treated i.t. with 10^7 plaque-forming unit (pfu) of rMVA-tmIgE, showed 24 and 48 hours after treatment a good infection efficiency. Moreover, the ability of rMVA-tmIgE to express human IgE *in vivo* was evident just one day after the intratumoral treatment, even if the signal of the Cε3Cε4 expression was much lower compared to the signal of MVA infection. This preliminary data is the first evidence that rMVA-tmIgE can be used to treat solid tumors in the mouse model "humanized" for the high affinity receptor of IgE ($Fc\varepsilon RI\alpha^{-/-}/hFc\varepsilon RI\alpha^{+}$). Indeed, our previous studies, based on preventive vaccination protocols, have shown the efficacy of rMVA-tmIgE in countering tumor

growth in the “humanized” mice model. In the near future we will determine the effect of i.t. rMVA-tmIgE in humanized mice carrying a solid tumor

We expect to achieve an inflammatory reaction against the tumor induced by the binding of IgE, expressed on tumor cells, with FcεRI receptor expressed on the surface of immune cells. We think that this initial IgE-dependent response would lead to long-term protection through the activation of an immune cascade where CD8+ T cells are surely involved.

INTRODUCTION

Immune System

The immune system defends body against infections elicited by foreign invaders such as virus or bacteria. Immune system consists of different cells, tissues and organs that collaborate together to recognize and eliminate agents potentially capable of disrupting the body's integrity. The key role of immune system is to distinguish the body's cells (self) from the foreign cells (non-self). In fact, it recognizes "self-marker molecules" expressed on self-cells, but when it encounters cells or organisms displaying "foreign-marker molecules", a response to eliminate them is rapidly triggered. Every molecule that activates this immune response is called "Antigen" (Ag), and it can be a part of a microbe, virus, cell or tissue from another person (with the exception of an identical twin) which is recognized as non-self.

The immune system employs two ways to protect body against infections: the innate immune system and the adaptive immune system.

The innate immune system is the first to intervene and involves different tools to respond to an infection. The main components of the innate immune system are represented by physical epithelial barriers, leukocytes, dendritic cells, and natural killer (NK) cells (Janeway CA Jr and Medzhitov R 2002). The epithelium that covers the respiratory tract and the gastrointestinal tract are our primary defense against microbial infection; in these tracts, mucous secretion by epithelial cells creates a physical obstacle to the passage of microbes, and antimicrobial chemical secretions by epithelial cells inhibit bacterial growth. Moreover, in the gastrointestinal tract many ingested microbes are killed or neutralized by stomach acid and digestive enzymes.

Cells included in the innate immune system are represented by leukocytes (a class of white blood cells) composed by Natural killer (NK) cells, mast cells (MC), eosinophils, and basophils; also phagocytic cells, such as macrophages, neutrophils and dendritic cells are included in the cell repertoire of innate immune system. All these cells derived from CD34+ pluripotent hematopoietic stem cells, present in the bone marrow (Bruce A et al. 2002). Leukocytes act identifying cellular debris or foreign particles, and eliminating pathogens that could cause an infection (Beutler B 2004). For example, NK cells kill virus-infected cells and cancer cells in nonspecifically way; they act by releasing proteins called "perforin" that produce pores in the cell membrane of target cells; pores induce apoptosis of target cells by favoring the entrance of serine proteases (also known as

granzymes) that cause cell death and thereby prevent the spread of viruses (Brandstadter JD and Yang Y 2011).
Otherwise, phagocytic cells are responsible for the production of chemokines and cytokines which trigger inflammation and other responses such as the induction of apoptosis and phagocytosis of infected cells (Beutler B 2004; Arango DG and Descoteaux A 2014). Moreover, macrophages and dendritic cells are also called APC (Antigen-presenting Cells), since they are able to present the antigen on their surface, through the MHC (Major Histocompatibility Complex) class II, and activate the adaptive immune system through the stimulation of T CD4+ lymphocytes (also known as T helper) (Banchereau J and Steinman RM 1998).
These mechanisms are always present and may enter in action at a remarkable speed. However, the innate immune system does not always have the power to eliminate the infection and it is not able to confer a long-lasting immunity against pathogens.

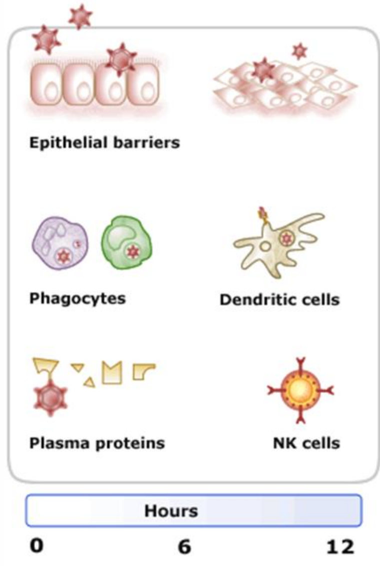


Figure 1. Main components of the innate immune system that are implicate in the first hours of immune response.

On the other hand, the adaptive immune system focuses specifically on defeating pathogens and evokes a long-lasting protection against that particular germ. In contrast to the innate immune response, which always possess the same repertoire of molecules to recognize the pathogens, the adaptive immune response uses millions of various immunoglobulins (Ig) and T cell receptor (TCR), each of which recognizes a different molecular structure (Cooper MD and Alder MN 2006). The cells that constitute adaptive immune system are formed in the bone marrow, where they often

ripen and subsequently migrate, through the blood and lymphatic system, towards the periphery to monitor tissues. These cells are called B lymphocytes (or B cells) and T lymphocytes (or T cells) and derived from hematopoietic stem cells of bone marrow (as leukocytes of innate immune system). Lymphocytes are unique cells which have specific receptors for the antigen and, therefore, represent the heart of adaptive immunity; even though both originate in the bone marrow, B and T lymphocytes differ in function, compartment where differentiation take place, and in the expression of different antigen receptors.

B lymphocytes grow in the bone marrow before encountering the antigen; subsequently, they move to the peripheral lymphoid tissues where interact with foreign antigens. Antigen binds and activates the receptor, represented by IgM and IgD, expressed on the surface of naïve B lymphocytes. This activation can be dependent or independent from T lymphocytes and leads to proliferation of the clone of B lymphocytes specific for the antigen, leads to their differentiation that consists in the generation of plasma cells that actively secrete antibodies and, finally leads to the production of memory B lymphocytes. Activated B cells may start to produce antibodies other than IgM and IgD, a defined class exchange process of heavy chains. The activation of B cells and the resulting antibody responses to antigens require the collaboration of T helper CD4 + cells able to recognize the same antigens. The definition of “T helper” cells derived from the fact that these cells "help" B cells to produce antibodies, stimulating B cells through different mechanisms. B lymphocytes, at this point, differentiate into cells which produce antibodies and in long survival memory cells. In fact, some plasma cells migrate from secondary lymphoid organs to the bone marrow, where they may remain for several years producing low levels of antibodies that provide long-lasting protection against specific antigen.

As we have mentioned, also the T cells are a part of the adaptive immune system. T lymphocytes arbitrate cell mediated immunity, indeed, their antigen-receptors recognize only peptides of antigens conjugated to molecules specialized to present the antigen, called MHC expressed on APC. T cells that express CD4 molecule are called T helper cells because they help both B lymphocytes to produce antibodies and phagocytes to destroy microbes embedded; instead, T cells that express CD8 molecule are called cytotoxic T cells (CTL) because they kill cells which possess intracellular microbes.

If the response by adaptive immunity is successful, it establishes a long-lasting protection against the pathogen that caused the response. A failure may be caused by immune deficiency or by the ability of pathogen to deceive the immune response (Parham P 2001).

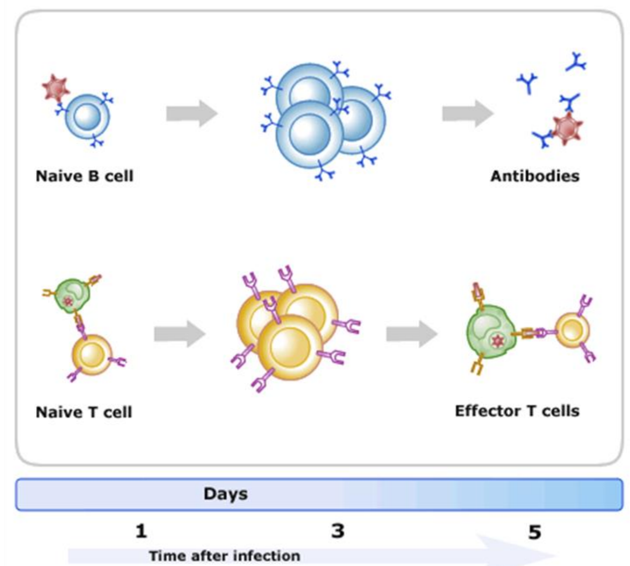


Figure 2. Main components of the adaptive immune system that enter in action against pathogens that are able to evade or overcome innate immune defenses.

Immunoglobulins

In 1890, von Behring and Kitasato temporarily immunized an animal to diphtheria by injecting blood serum of another animal infected with this germ. They demonstrated that this serum had not only preventive but also curative properties since when injected at the first symptoms of diphtheria, it induced the healing of the affected animal. Through this study, Behring and Kitasato discovered an agent present in the blood which was capable to neutralize diphtheria toxin: this agent was the immunoglobulin. After 100 years, the study of the structure and function of immunoglobulins, highlights the complex nature and importance of these proteins.

Immunoglobulins are glycoproteins with a quaternary structure, formed by two heavy chains (H) and two light chains (L); each chain consists of variable (V) and constant regions (C) with immunoglobulin domains, covalently linked by disulfide bonds, electrostatic interactions and Van Der Waals forces. Each immunoglobulin domain contains about 100 amino acids arranged in a β structure, formed by 3 and 4 β sheets (William AF and Barclay AN 1988). The light and heavy chains variable regions constitute two identical binding sites for the antigen which, together with the adjacent constant domains, form the antibody Fab region. The remaining immunoglobulin domains form, instead, the Fc region of the antibody that contains the binding domains for the FcR receptor.

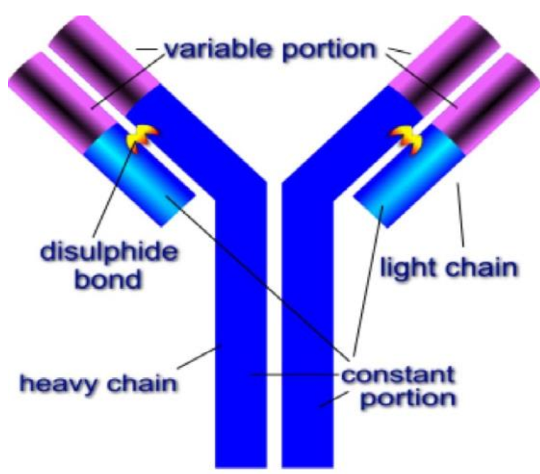


Figure 3. Immunoglobulin structure.

Within the variable domains, there are three regions characterized by a much higher variability than the rest of the domain, called hyper variable regions or complementarity-determining regions (CDR1-3). These regions have different sequences in different immunoglobulins, determining the specificity of the binding during the antibody-antigen recognition. The light chains are divided into two classes called λ, and κ; the heavy chains are divided into five different classes called μ, δ, γ, α and ε enabling the division of immunoglobulin in as many isotypes called IgM, IgD, IgG, IgA, and IgE, characterized by different effector properties (Bengten E et al. 2000). There is also another subclassification for IgG and IgA, respectively γ1, γ2, γ3, γ4 and α1, α2 (Woof JM and Burton DR 2004). The different classes of heavy chains are capable to combine with both light chains, but the number of constant domains is that diversifies, at the structural level, the different isotypes of immunoglobulins. IgD, IgG and IgA have, in fact, three constant domains C_H, whereas IgM and IgE possess four constant domains. There is also, between the first and the second C_H domain, a hinge region rich in proline, serine and threonine, that gives to the region highly flexible properties; this flexibility is essential to modulate the distance between the two binding sites for the antigen present in the antibody.

The Generation of Antibody Diversity

Immunoglobulin form a part of B lymphocytes receptor (BCR); immunoglobulins, as population, can bind an infinite number of ligands (antigens) totally different one from each other, and this ability is given by a complex series of mechanisms that alter DNA of each B cell.

During our life, the immune system encounters thousands of different antigens from bacteria, viruses and other microorganisms and this raises the question on how antibody diversity is generated during development. Nature has invented an ingenious solution to overcome this problem: the combinatorial design of the variable regions of antibodies.

During maturation of the immune system, antibodies are generated by random fusion of the variable segments of the light and heavy chains through a process called V(D)J-recombination, that is catalyzed by the DNA recombinases RAG-1 and RAG-2.V(D)J recombination takes place in the bone marrow for B cells and in the thymus for T cells, and in a almost random manner rearranges variable (V), joining (J), and in some cases, diversify (D) gene segments (Hoehn KB et al. 2016).

In the germline, the genetic locus of the light chain consists of up to 40 V-segments and 5 J-segments; the heavy chain contains 50 V-segments, 27 D-segments and 6 J-segments.

For the light chain, one V-segment gets fused to one of the J-segments by recombination. The production of the variable heavy chain occurs in two steps, in the first step one of the J-segments is fused to the D-segment, and, later the combined DJ-sequence is added to one of the V-segments. The recombined antibody gene is transcribed together with the constant region of the antibody that is added as exon by splicing, and finally, heavy and light chains are assembled into a functional antibody. Stability of the secreted antibodies is reached by covalent disulfide bonds between heavy and light chains. Since each antibody is generated by combinatorial fusion of one of 40 V-segments, 5 J-segments for the light chain and one of 50 V-, 27 D- and 6 J-segments for the heavy chain, the number of possible combinations amounts to 1.6×10^6. Furthermore, during the recombination process additional mutations are introduced. This increases the number of possible rearrangements to more than 10^{10} (Hoehn KB et al. 2016).

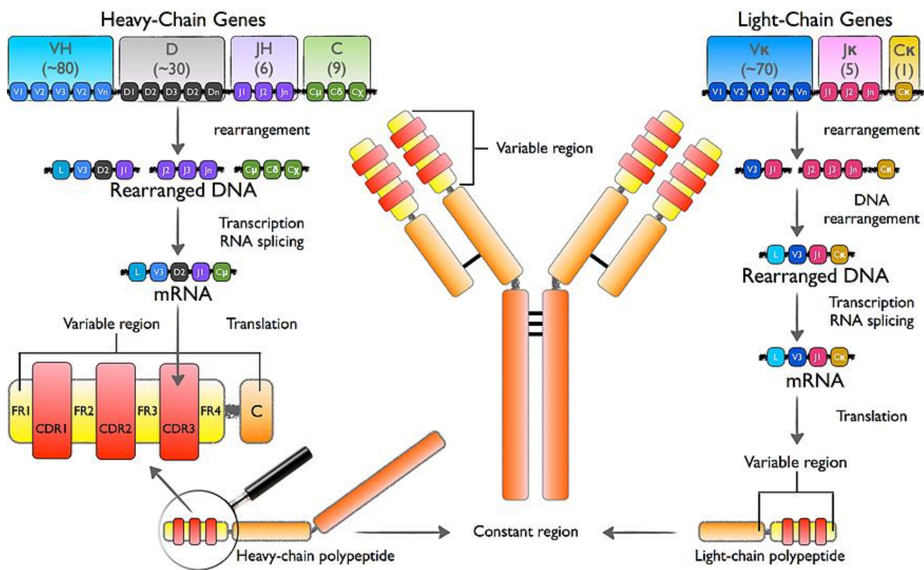


Figure 4. VDJ rearrangement to assemble a functional and specific immunoglobulin.

These events lead to the expression of a single type of H and L chain in each B cell; as a consequence of these events, in a single B cell and in its progeny is expressed only one type of immunoglobulin with a single antigen specificity. In a recent study it was calculated that the number of possible BCR sequences is enormous, at least 10^{18} (Elhanati Y et al. 2015), much higher than the number of B cells present in the body.

Sometimes, the V(D)J recombination can generate inactive recombinant sequences that cause B cell apoptosis; the remaining cells, naïve B lymphocytes, are subjected to a first round of selection for the lack of self-reactivity prior to being released from the bone marrow (Janeway CA Jr et al. 2001). The biological development of B cells is therefore characterized by a first phase with an antigen-independent clonal expansion of the progenitors followed by a second phase distinguished by maturation, induced by the antigenic stimulus leading finally to the formation of mature plasma cells and memory B cells.

Initially, immunoglobulins expressed on the membrane (membrane immunoglobulin, mIg) are produced and these bind their specific antigens, but, this interaction alone is not able to activate intracellular signals that should follow the link with Ag; in fact, the cytoplasmic portions of the mIg molecule are very short and are not able to interact with intracellular proteins. The mIg-Ag interaction, occurring on the membrane, are communicated within the cell by two trans-membrane proteins, called Igα and Igβ that are associated with membrane Ig. These auxillary proteins have an extracellular portion and a cytoplasmic tail containing the ITAM motif

(Immunoreceptor Tyrosine-based Activation Motif) involved in intracellular signalling; therefore, the functional B cell receptor (BCR), is in fact a complex formed by the mIg, Igα and by Igβ proteins (Abbas AK et al. 2002).

Following alternative splicing of mRNA, the C-terminal domainof Ig is changed and this leads to secretion of Ig (soluble immunoglobulin, sIg) characterized by the same specificity of the mIg (Gould HJ et al. 2003). This event also involves the modification of functional Ig because the Fc portion of the sIg is able to interact with receptors expressed on effector cells leading to activation of the mechanisms responsible for antigen elimination.

IgE: structure and functions

IgE consist of 2 heavy (ε) and 2 light (κ or λ)chains; they are characterized by the ε type heavy chain that contains one variable region V_H and 4 domains of the constant region, Cε1-4. The extra domain (Cε2) present in IgE appears to be critical regarding the peculiar physical properties and specific functions of this antibody class, in fact, the Cε2-4 Fc domains confer its isotype-specific functions, including binding to its receptors FcεRI and FcεRII (also known as CD23).

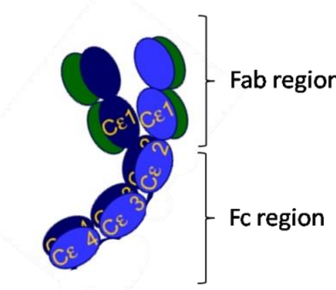


Figure 5. Structure of IgE.

IgE possess cool features, as they are able to induce a rapid and strong pathologic response and to act as a highly sensitive amplifier which causes immunological reactions after the recognition of small amounts of antigen even with a small number of IgE. Many aspects of IgE immunobiology are interesting, for example its specific cellular receptors, the effector cells that mediate its functions, and its immunomodulatory properties (Oettgen HC 2016).

IgE is an evolutionary conserved member of the immunoglobulin family. Normally, in the serum of healthy individuals, all type of Ig are presents in concentration of micro- to milligrams per ml; conversely, the titre of IgE is very low compared to others Ig, with a range of nano- to micrograms

per ml (Gould HJ et al. 2003) and this concentration reflects the number of circulating B cells involved in IgE synthesis. IgE levels are influenced by age, immune status, environmental factors and by the presence of diseases.

Compared to Immunoglobulin G (IgG), which has a half-life of approximately 3 weeks, the half-life of IgE is very short. In plasma, the half-life of IgE is less than one day, but, when IgE binds its receptor it can remain fixed on mast cell which expressed the receptors for IgE; for this reason IgE can remain in tissues for weeks or months, and this long half-life in tissues has considerable clinical implication. An example may be given by the passage of sensitivity to an antigen (for example towards peanuts) from an organ donor to its recipient mediated by mast cell in which IgE linked its receptor.

IgE are physiologically involved in the immune response against intestinal parasites and physiopathologically in allergic responses. Although parasites, such as helminths, activate different innate immune mechanisms, these organisms are often able to survive and replicate inside their host; as a first line of defense, neutrophils and macrophages attack the worms secreting microbicides that allow to kill microbes that are too big to be swallowed from phagocytes.

Following exposure to an allergen, IgE are capable to trigger an allergic immune response even after a few minutes of exposure (type I hypersensitivity) and during this event, IgE activate mast cells and basophils, causing degranulation of these cells and release of preformed mediators such as histamine, heparin, proteases, and chemokines from cytoplasmic granules (Karagiannis SN et al. 2012; Leoh LS et al. 2015)and provoking an acute local inflammation (Gould HJ et al. 2003; Gould HJ and Sutton BJ 2008). A new exposure to the same allergen leads to a more rapid activation of mast cells and basophils, resulting in the recruitment of inflammatory cells such as neutrophils, eosinophils, monocytes/macrophages, and T cells (Ying S et al. 1999).

IgE receptors

The biological functions of IgE antibodies are mediated by their interaction with specific antigens and different receptors. There are two IgE receptors, a high-affinity FcεRI receptor and a low affinity FcεRII receptor; both of these receptors are proteins expressed on the effector cells membrane and are able to specifically bind to the constant region Fc of IgE. As IgE, also other antibody isotypes bind their specific receptors present on different cells of immune system, and this binding leads to different effector functions including phagocytosis, antibody-dependent

cytotoxicity (ADCC), and modulation of cellular secretion of preformed mediators (Metzger H 2002).

The High-Affinity IgE Receptor (FcεRI)

The high-affinity IgE receptor FcεRI is a multimeric protein expressed in two isoforms, a tetrameric αβγ2 and a trimeric αγ2 receptor. In humans, the FcεRI receptor is expressed in large amounts (about $2x10^5$ molecules per cell) on the mast cells and basophils surface, and, at lower levels, on Langerhans cells, monocytes, platelets and eosinophils surface.

The classical structure of the FcεRI receptor is tetrameric and consists of an α chain required for binding to IgE, a β chain necessary for the maturation of the receptor and for an increase of its expression on the cell surface and two γ chains, linked by a disulfide bridge, involved in signal transduction (Donnadieu E et al. 2000). The α chain is constituted by an amino-terminal extracellular region, a transmembrane region and a cytoplasmic tail. In particular, in the extracellular region, there are two glycosylated immunoglobulin domains D1 and D2 which constitute the binding site for IgE (Garman SC et al. 2000). The β chain consists of four transmembrane domains separated by intra and extracellular loops. The β and γ chains have intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) able to mediate the association with proximal kinases (Kinet JP 1999).

In humans, the tetrameric receptor (αβγ2) is constitutively expressed on effector cells of anaphylaxis (mast cells, basophils and eosinophils), while the expression of the trimeric form of FcεRI (αγ2) is present on antigen presenting cells (APCs) such as monocytes, dendritic cells and Langerhans cells (Kinet JP 1999). Despite the lack of the β chain, the function of the receptor on APCs is intact, even if the expression density corresponds of about 1/100 compared to that in basophils. The expression of FcεRI is influenced not only by the presence of the β subunit but also by the local concentration of circulating IgE; in fact, the IgE binding to the receptor constitutes a positive signal for the conservation of the receptor on the cell surface (MacGlashan D Jr 2005).

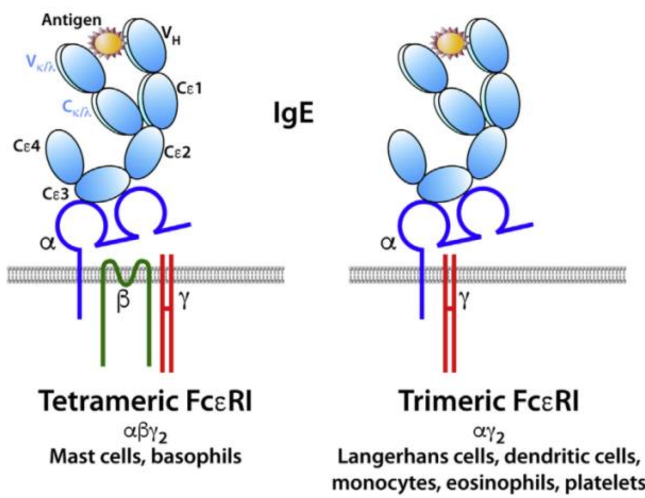


Figure 6. Interaction between tetrameric FcεRI and IgE or trimeric receptor and IgE.

Differently from humans, the expression of the receptor in mice has been detected exclusively on the surface of mast cells and basophils in the classical tetrameric αβγ2 form (Kinet JP 1999). However, FcεRI is present on rat eosinophils, monocytes/macrophages, and platelets (Dombrowicz D et al. 2000; Gould HJ and Sutton BJ 2008).

FcεRI activation

The affinity of IgE to FcεRI (Ka = 10^8 - 10^{11} M^1) is 2-5 orders of magnitude higher than that of IgG to their receptors. In addition, the high affinity of IgE-FcεRI complex makes that IgE is the only class of antibodies that can be strongly retained by the effector cells in the absence of antigen and explains the long half-life of IgE in the tissues (about 2 weeks in the skin measured), which it is longer than that measured for IgG (2-3 days) (Penichet ML and Jensen-Jarolim E 2010).
The role played by FcεRI in allergic reactions, both in the "initial phase" which involves mast cell activation and degranulation, and in the "late stage", which involves the recruitment and activation of inflammatory cells, have been extensively researched.
In hypersensitivity reaction, allergen induced cross-linking of IgE bound by FcεRI triggering a cascade of signaling events, resulting in the release of mediators and gene transcription. Cross-linking of neighboring FcεRI receptors leads to aggregation and transphosphorylation of cytosolic ITAMs on the FcεRIβ- and γ-chains by constitutively receptor-associated Lyn tyrosine kinase. The initiation of downstream signaling pathways is characterized by an increase in cytosolic calcium,

activation of gene transcription of IL-4, TNF, and IL-6, synthesis of cysteinyl leukotrienes and prostaglandins and in the fusion of granule with the plasma membrane, followed by the release of preformed mediators of hypersensitivity. The release of mediators, during an hypersensitivity reaction, may causes vasodilatation, plasma extravasation, tissue edema, mucus production, and smooth muscle constriction.

Subsequently to this initial response, local or recruited IgE-sensitized APC encourage B cells to produce IgE, thus maintaining mast cells and APC sensitization and long-term immunologic memory and immune surveillance both in situ and systemically.

The low-affinity IgE receptor (CD23)

The affinity of IgE to CD23 is characterized by a Ka = 10^{7}–$10^{8}M^{-1}$ and, unlike FcεRI, the low affinity receptor CD23 (FcεRII) does not belong to the immunoglobulin superfamily; it is in fact classified as a type 2 of transmembrane protein characterized by a calcium dependent lectin domain, present in the distal region of the extracellular domain (C-terminal). CD23 receptor is present in two variants, CD23a and CD23b that differ in seven and six amino acids, respectively, at the N-terminal cytoplasmic domain level, which contains different signals that can modify their functions (Yokota A et al. 1988). CD23a is expressed on antigen-activated B cells prior to differentiation into antibody-secreted cells and is involved in IgE antibody-dependent antigen endocytosis, processing, and presentation (Gould HJ et al. 2003). The expression of human CD23b isinduced by IL-4 on various inflammatory cells as T cells, eosinophils, platelets, monocytes, B cells, DC and epithelial cells.

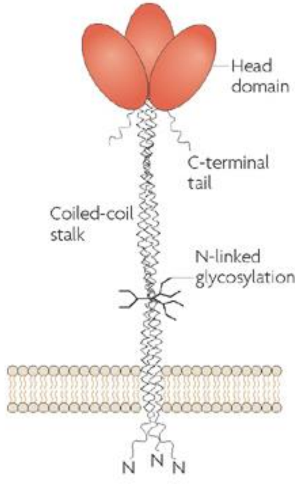


Figure 7. Structure of CD23

The CD23 receptor is involved in various processes including the IgE-dependent presentation of antigen in human and murine B cells, the inhibition of IgE synthesis in murine B cells through a negative feedback mechanism, and the IgE-dependent cancer cells killing by the activation of human monocytes (Karagiannis SN et al. 2003). The soluble fragments of the receptor may also, depending on their structure, regulate positively or negatively IgE synthesis in human B cells.

Allergy and Tumor

In the last decades, we have seen a continuous increase in allergies, especially in the industrialized world, so much so that it has been proposed a possible involvement of allergy in various diseases such as diabetes mellitus, cardiovascular diseases and cancers (Gergen PJ et al. 2000).

Regarding cancers, it has been proposed that the predisposition to allergic phenomena may play a protective role or constitute a risk factor. Since the allergy is an hyper-reactive condition of immune system that directs T cells toward a Th2 response, it was assumed that the increased immune-surveillance could contrast the proliferation of tumor cells; conversely, the allergic condition responsible in some cases of a chronic inflammation resulting in tissue damage and repeated shelter, could increase the risk of cancer in certain tissues. For example, the increase of lung cancer in asthmatic individuals who have never smoked, supports a direct relationship between asthma and lung cancer (Santillan AA et al. 2003).

The emerging interdisciplinary field of *AllergoOncology* represents a multifaceted attempt to determine the inter-relations of cancer and the Th2 branch of the immune system, which has recently greatly expanded from clinical observations to an understanding of the molecular mechanism leading to improve targeted therapies.

Despite numerous studies, it is difficult to draw a precise picture of the association between allergies and cancer, often in fact, the results are conflicting with problems associated to the low number of subjects with a specific allergy subtype (Turner MC et al. 2006); another complication is represented from the patients with neoplasms and immunocompromised, condition that leads to an attenuation of the allergic symptoms and the immune system response. Several epidemiological studies have investigated a possible association between allergies and other types of cancer. In some cases it was demonstrated a protective role of allergies to certain types of cancer, for example, a decreased risk associated with a history of hives are seen in males for oral

cancer and in females for tumors of digestive system (Vena JE et al. 1985); in many cases, the risk of childhood leukaemia tends to be lower in atopic people. In other cases, the allergic predisposition was seen instead as a risk factor for glioma (Wang H and Diepgen TL 2005).
Regarding mechanisms, the overall working hypothesis is that allergens may crosslink IgE antibodies fixed to effector cells by means of allergen-associated molecular patterns (AAMPs), analogous to tumor antigens by tumor-associated molecular patterns (TAMPs). The immune response is, on the one hand, inappropriate hypersensitivity toward innocuous allergens, but on the other hand is the activation of anti-cancer inflammation; the final result depends on which type of effector cells will be involved (Jensen-Jarolim E and Pawelec G 2012).
The scenario that is anyway emerging from most epidemiological data indicates that allergic predisposition may be associated with a reduced risk for pancreatic cancer, childhood leukemia (Turner MC 2012) and for brain tumor (Brenner AV et al. 2002). Conversely, asthma would prove to be a risk factor for the onset of lung cancer; this potential positive association could be due to many factors such as high levels of free radicals and reduced levels of antioxidants agents in the respiratory tract (Boffetta P et al. 2002) and continuous stimulation of cell regeneration for the shelter of the inflammatory tissue damage.

IgE in tumor immunotherapy

Even if the physiological role of IgE has not been completely clarified yet, it was observed that this class of immunoglobulins can have a role in cancer prevention. As mentioned above, the concentration of IgE is very low in physiological condition but can dramatically increase in infections and allergic reactions; epidemiological and experimental studies showed that these conditions are negatively correlated to the development of tumors (Turner MC et al. 2006). For instance, the development of a syngeneic adenocarcinoma of mice was abolished in *Nippostrongylus brasiliensis* infected mice whether the infection was given before the tumour inoculation, and infected mice had less probability to develop spontaneous mammary carcinoma (Ogilvie BM et al. 1971).
These findings have led to the hypothesis of a possible exploitation of the activity and characteristics of IgE in tumor immunotherapy. Some studies conducted to investigate a possible use of IgE in anti-tumor therapy have come to the conclusion that the redirection of IgE to tumor antigens is extremely efficient in triggering an anti-tumor effect (Leoh LS et al. 2015). The ability to

activate immune responses that may be directed against tumor is the cause of the success of the use of antibodies in cancer therapy. The advancement of technology along with experience in clinical applications have enabled researches to design anti cancer antibodies more efficiently and it also allowed to study a new class of antibodies as IgE that can be used in addition to that already employed in clinical, represented by IgG. IgE have a crucial role in allergic reactions and possess several properties that may be advantageous to counteract tumor growth; there are numerous studies performed to date that involve IgE in immune therapy against cancer and that demonstrate the efficacy of passive and active immunotherapeutic approaches (Leoh LS et. al 2015).

IgE species	IgE specificity	Route of administration	Targeted cancer cells (route of cell inoculation)	Mouse model	References
Mouse	gp36 of MMTV	i.p.	H2712 mouse mammary carcinoma (s.c. and i.p.)	C3H/HeJ	Nagy et al. (1991)
Rat/human chimeric	Mouse Ly-2	s.c.	E3 mouse thymoma (s.c.)	C57BL/6	Kershaw et al. (1996)
Mouse	DNP	i.p.	MC38 mouse colon carcinoma cells expressing human CEA (s.c.)[a]	C57BL/6	Reali et al. (2001)
Mouse	DNP	s.c.	TS/A-LACK mouse mammary carcinoma cells coated with DNP (s.c.)	BALB/c	Nigro et al. (2009)
Mouse and mouse/human chimeric	Colorectal cancer antigen	i.v.	Human COLO 205 (s.c.)	SCID	Kershaw et al. (1998)
Rat/human chimeric	Mouse Ly-2	i.p.	E3 mouse thymoma (i.p.)	NOD–SCID	Teng et al. (2006)
Mouse/human chimeric	FBP	i.v.	IGROV-1 human ovarian carcinoma cells (s.c.)	C.B-17 scid/scid	Gould et al. (1999)
		i.p.	HUA patient-derived ovarian carcinoma (i.p.)	nu/nu	Karagiannis et al. (2003)
Mouse/human chimeric	NIP	s.c.	TS/A-LACK mouse mammary carcinoma cells coated with NIP (s.c.)	Human FcεRIα Tg BALB/c	Nigro et al. (2009)
Human	HER2/*neu*	i.p.	D2F2/E2 mouse mammary carcinoma cells expressing human HER2/*neu* (i.p.)	Human FcεRIα Tg BALB/c	Daniels et al. (2012a)
Human (truncated)	N/A	s.c.	TS/A-LACK mouse mammary carcinoma cells coated with truncated IgE (s.c.)	Human FcεRIα Tg BALB/c	Nigro et al. (2012)
Mouse/human chimeric	MUC1	s.c.	4T1 tumor cells expressing human MUC1 (s.c.)	Human FcεRIα Tg BALB/c	Teo et al. (2012)
Mouse/human chimeric	PSA	s.c.	CT26 tumor cells expressing human PSA (s. c.)	Human FcεRIα Tg BALB/c	Daniels-Wells et al. (2013)

Table 1. Summary of IgE antibodies and mouse models used to evaluate their anti-tumor efficacy (from Leoh LS et al. 2015).

Immunotherapy against cancer aims to restore the reactivity of the host's immune system to fight cancer and can be divided into two types: passive and active immunotherapy.

Passive cancer immunotherapy provides a tumor antigen-specific immune response by supplying high amounts of effector molecules. Examples of passive immune therapies are those based on the use of monoclonal antibodies (mAb) directed against tumor antigens conjugated to toxins,

radioisotopes or drugs, and immunocompetent cells such as LAK (Lymphokine Activated Killer) and TIL (Tumor Infiltrating Lymphocyte) that is defined adoptive cellular immunotherapy.
In contrast, active cancer immunotherapy aims at induction of an endogenous, long-lasting tumor antigen-specific immune (preventive or therapeutic) response, and consists of the establishment and expansion of the endogenous immune cells repertoire (Riemer AB et al. 2007). This approach is based on the administration of tumor antigens in an individual in order to activate the immune effectors cells; an alternative to induce an immune response *in vivo* (by administration of a tumor antigen as a vaccine to the host's APCs) is *ex vivo* stimulation of autologous dendritic cells (DCs) with re-application of the DCs to the patient, as a method to break the patient's immune tolerance for tumor-associated antigen.

IgE in passive immunotherapy

In 1991 Nagy and collaborators, using hybridoma technology, have developed IgE-producing hybridomas from splenocytes of Balb/c mice challenged with mouse mammary tumor virus (MMTV). Repeated administration of anti-gp36 mAbIgE (i.p. injection of 25 µg of anti-gp36 mAbIgE every 4 days, for 8 weeks) in syngeneic mice C3H/HeJ, previously challenged s.c. with 10^5 or 10^6 of mammary adenocarcinoma MMTV-secreting cells, prevented tumor development in 50% of the animals. The antibody had shown no effect on the growth of a MMTV-negative tumor control, supporting the idea that a mechanism of cytotoxicity mediated by antigen-specific IgE could play a role of anti-tumor immune surveillance (Nagy E et al 1991).
In 1996, it was produced a second tumor-specific IgE antibody, in this case specific to Ly-2 expressed on murine T-cell tumors (Kershaw MH et al. 1996). This antibody was a mouse/human chimeric IgE and, since human IgE does not bind the murine receptor FcεRI (Kinet JP 1999), in this study it was employed a chimeric receptor composed by the human FcεRIα extracellular domain linked to transmembrane FcγRIIa and the cytoplasmic tail of the CD3ζ signaling molecule; this chimeric receptor was called 3H2.
In vitro assay showed that mouse CTL effector cells, expressing the chimeric receptor 3H2, were able to destroy the mouse E3 thymoma cells that express Ly-2, if these cells were pre-incubated with mouse/human anti-Ly-2 IgE. Moreover, if 3H2 effector cells were incubated with E3 thymoma cells in presence or absence of mouse/human anti Ly-2 IgE, and then injected s.c. in mice, the results showed that the anti-tumor effect was more evident in the presence of mouse/human

anti-Ly-2 IgE; in fact, only 1 mouse out of 5 animals developed tumor compared to 4 out of 5 mice treated with 3H2 cells alone (Kershaw MH et al. 1996).

Kershaw and collaborators developed another mouse monoclonal IgE antibody (mIgE 30.6) with the purpose to determine a possible antitumor effect of IgE (Kershaw MH et al. 1998). This mouse antibody was specific to an antigenic determinant expressed on the surface of human adenocarcinoma of the colon-rectal cells, including COLO 205. The study showed that the intravenous administration of 1µg of mIgE 30.6 was able to significantly inhibit the growth of COLO 205 cells, implanted subcutaneously in SCID mice; in this case, the administration was carried out 5 days after tumor cells implantation. Unfortunately, the effect was not boosted by repeated administration of the antibody (Kershaw MH et al. 1998).

Another study was performed on nude mice challenged i.p. with human HUA ovarian cancer cells, expressing FBP (Folate binding protein), and then treated i.p. with human PBMC alone or in combination with MOv18-IgE or MOv18-IgG1 (Karagiannis SN et al. 2003). MOv18-IgE is a mouse/human chimeric IgE specific to FBP (Gould HJ et al. 1999) similar to MOv18-IgG1 (mouse/human chimeric IgG1) that containing the same variable regions. This study demonstrated that MOv18-IgE significantly increased the survival of mice up to 40 days compared to MOv18-IgG1 treated mice that showed a survival of 22 days. Since HUA ovarian cancer cells derived from a human primary tumor passed in mouse, this xenograft model mimics more closely a possible clinical situation. Moreover, with this study it was demonstrated that human monocytes, infiltrated into the tumor, exert IgE ADCC (Antibody-Dependent Cell-mediated Cytotoxicity) against tumor cells.

It is through two distinct pathways that monocytes are able to mediate, *in vitro*, the destroy MOv18 IgE-dependent of ovarian cancer cells: by cytotoxicity and by phagocytosis (Karagiannis SN et al. 2007). The study of the specific role played by the receptors for IgE has identified in FcεRI the mediator of ADCC and in CD23 the mediator of ADCP (Antibody-Dependent Cell-mediated Phagocytosis).

Her2/neu (human epidermal growth factor receptor 2) is over expressed in 20-30% of all breast and ovarian cancers and is associated with a poor prognosis. The antitumor effect of IgE antibody specific for Her2/neu was evaluated; Her2/neu is a member of the EGFR family and mediates cell survival and proliferation (Martinelli E et al. 2009).

For the treatment of metastatic breast cancer, it was evaluated the efficacy of Trastuzumab (Herceptin©), a humanized IgG1 but, even if the effect is evident both employed Trastuzumab alone or in combination with chemotherapeutic agents, most of the treated patients showed relapse and many patients, instead, do not show any response to therapy based on humanized IgG1 (Ahn ER and Vogel CL 2012); this has prompted to seek new strategies to treat tumors over expressing HER2/neu.

In a 2009 study, Karagiannis and colleagues have produced a humanized trastuzumab IgE, resulting from the fusion of the original trastuzumab variable regions with the constant region of the human IgE; they demonstrated that both antibodies showed, *in vitro*, cytotoxic effects in cell viability test using SK BR-3 human breast cancer cells; moreover, the effects was observable for 2 days. Trastuzumab IgE mediated ADCC in the presence of U-937 human monocytic cells and, degranulation was triggered by trastuzumab IgE using murine colon carcinoma cells expressing human Her2/neu (CT26-Her2/neu) incubated with RBL SX-38, a rat basophilic leukemia cells that express human FcεRI (Karagiannis P et al. 2009).

Subsequently, it was developed a fully human anti-Her2/neu IgE antibody that induced, *in vitro*, degranulation of RBL SX-38 in the presence of murine mammary carcinoma cells expressing human Her2/neu (D2F2/E2) but was not detected any degranulation in presence of D2F2 cells which lack the expression of Her2/neu (Daniels TR et al 2012a). The study suggests that a type I hypersensitivity may occur in close proximity of the tumor in which Her2/neu is over expressed on the surface of tumor cells and this can facilitates FcεRI cross-linking and the activation of degranulation by effector cells.

Very interesting is also the development of the mouse/human chimeric IgE specific for human MUC1 (Teo PZ et al. 2012) over expressed on tumors arising from glandular epithelium, such as breast, ovary, pancreas, and colon cancers. The anti-tumor effect of anti-MUC1 IgE was evaluated using 4T1.hMUC1, a murine breast carcinoma cell line expressing the transmembrane form of human MUC1; in mice challenged subcutaneously with 10^5 of 4T1.hMUC1cells and then treated with 20µg anti-MUC1 IgE for 5 days, it was observed a 24% reduction in tumor volume. Moreover, the treatment with anti-MUC1 IgE showed an increased in the presence of MC in necrotic peritumoral regions accompanied by degranulation, compared to mice treated with a control antibody. The fact that only in 24% of the mice was observed tumor reduction could be explained by the fact that the 4T1 tumors are not vascularized and this could prevent the correct penetration of the drug and the effector cells into the tumor mass.

Finally, IgE were used against prostate cancer (PCa) (Daniels-Wells TR et al. 2013). Prostate cancer is among the most diagnosed cancers the last few years. The prostate specific antigen (PSA) is a good target to employed in the immunotherapy against PCa. For this reason it was designed a mouse/human chimeric anti-PSA IgE containing the variable regions of the murine monoclonal antibody IgG1 AR47.47, specific for PSA. In the study, AR47.47-PSA showed improved antigen presentation by human dendritic cells and the activation of both TCD4 and TCD8 lymphocytes (Berlyn, K.A et al. 2001). Moreover, anti-PSA IgE-PSA complexes induced an immune activation and prolonged the survival of human FcεRIα transgenic mice challenged with PSA-expressing tumors in a prophylactic vaccination setting.

IgE in active immunotherapy

An alternative strategy to the passive immunotherapy based on IgE is to induce anendogenous IgE response; the oral immunization of mice with mimotopes of trastzumab (mimic epitopes recognized by the anti-Her2/neu trastuzumab), in neutralization and suppression conditions of gastric acid, resulted in the formation of specific IgE antibodies for the antigen Her2/neu. It was also demonstrated that theseIgE are able to activate, *in vitro*, effector cells mediating the lysis of tumor cells (Riemer AB et al. 2007).

To investigate the IgE-driven immune response in the prevention and control of cancer, our group has loaded non-specific monoclonal IgE on the surface of cancer cells through the 3-step mechanism (Reali E et al. 2001). The IgE loading mediated by a biotin-avidin bridge was performed both *in vivo*, by systemic administration of reagents such as biotinylated mAb anti-TAA avidin and biotinylated IgE mAb in C57BL/6 mice pre-challenged with tumor cells, and *in vitro*, on the same type of tumor cells, prior to inoculation. Exploiting both loading mechanisms, it has been shown that the presence of non-specific IgE on tumor cells surface, strongly affects tumorigenicity giving rise to a tumor-specific acquired immunity through priming of CD8+ and CD4+ T cells.
The loading with IgE, unlike that of control IgG, in addition of causing a delay in tumor growth and a significant decrease of the speed of tumor development, also confers protective immunity against subsequent challenges with untreated tumor cells. This has been seen in two different tumor models: the lymphoma cell line RMA characterized by fast growing, known to be immunogenic, and a slightly immunogenic colon adenocarcinoma cell line called MC38.

For the development of the biotin-avidin bridge, it has been exploited, in the first model the presence of the tumor antigen stably transfected Thy 1.1, while in the second model the CEA (Carcino embryonic Antigen).

The presence of IgE triggered an inflammatory reaction at the tumor site level through the recruitment and activation of the FcεRI receptor-expressing effector cells such as eosinophils, which promoted the destruction of tumor cells, an essential requirement for an efficient priming of the specific immune response dependent on CD4+ and CD8+ T cells. The role of eosinophils and CD4+ and CD8+ T cells in tumor-specific immune response driven by IgE has been proven through experiments of cellular depletion. The depletion of eosinophils,CD4+ and CD8+ T cells in mice prior to the injection of tumor cells loaded with IgE demonstrated, in fact, the abolition of the IgE driven anti-tumor effect. Finally, preliminary experiments of vaccination, confirmed that this IgE driven immunity, mediated by T cells, could actually be used to prevent cancer. The presence of IgE on the surface of irradiated tumor cells, in fact, conferred protection in both tumor models at doses 100 times lower than the corresponding control cells loaded with IgG (Reali E et al. 2001).

The adjuvant effect of IgE-coated tumor cells was later confirmed using a slightly different strategy; our group studied the effect of mouse IgE in anti-tumor protection by vaccination of mice with tumor cells loaded with IgE and infected with Vaccinia Virus. In this study, the introduction of Vaccinia Virus and MVA (Modified Vaccinia Virus Ankara) was done for several reasons: 1- to avoid the need to kill tumor cells by irradiation (vaccinia virus kill tumor cells in a few days); 2- to use the high immunogenicity of vaccinia; and 3- to determine a system suitable to induce cell surface IgE expression. Loading tumor cells with IgE was conducted by haptenization followed by loading of hapten-specific IgE; this method has allowed to apply the strategy to tumors not characterized by specific tumor-associated antigens (TAA) or slightly immunogenic.

C57BL/6 mice were immunized with loaded with IgE and infected with Vaccinia Virus; as controls were used infected RMA cells not loaded with IgE; fifteen days after immunization mice were inoculated with live tumor cells. In terms of percentage of survival, 60% of mice immunized in the presence of IgE, survived to inoculation of tumor cells not developing tumors even in subsequent months; in contrast, 100% of immunized mice in the absence of IgE has developed tumors.

Moreover, to demonstrate that the anti-tumor adjuvant effect of IgE was due to the interaction between IgE and its high affinity FcεRI receptor, we vaccinated *wild type* mice and αFcεRI *knock-out* mice (Dombrowicz D et al. 1993) with TS/A-LACK cells ($H\text{-}2^d$ mammary adenocarcinoma of

BALB/c origin) infected with MVA and loaded with IgE through haptenization. By monitoring the tumor growth after inoculation of live TS/A-LACK cells, it was observed that the anti-tumor protection observed in *wild type* mice in the presence of IgE, was completely lost in αFcεRI *knock-out* mice. In these mice, indeed, tumor growth was not influenced by the presence or absence of IgE and was comparable to that obtained in wild type mice in absence of IgE; this was the first demonstration of the crucial role played by the high affinity receptor FcεRI in anti-tumor effect driven by IgE (Nigro EA et al. 2009).

To test the anti-tumor adjuvant effect of human IgE,weemployed a transgenic mouse model FcεRIα$^{-/-}$hFcεRIα^{+},knock-out for the α chain of the murine FcεRI receptor and knock-in for the human α chain (Dombrowicz D et al. 1996). As for experiments with murine IgE, mice were vaccinated with the cellular vaccine in the presence or absence of human IgE and they were subsequently challenged with live tumor cells and monitored for tumor growth. In the murine model with human receptor, two immunizations with cells infected with MVA were able to give anti-tumor protection in the presence or absence of human IgE. With a single immunization, instead, IgE showed their adjuvant effect by ensuring an anti-tumor protection comparable to that obtained with two immunizations. Immunized mice with cells infected with MVA in the absence of IgE, in fact, were not protected whenchallengedwith live tumor cells, showing a tumor growth comparable to that of non-immunized control mice.

Finally, in the model with the murine receptor, a single immunization in the presence of human IgE, unlike what happened with the mouse IgE, was not able to give anti-tumor protection, since tumor growth was comparable to that observed in non-immunized control mice; this was due to the fact that the murine receptor was not able to bind human IgE. With this study, our group demonstrated the adjuvant effect of human IgE in the anti-tumor vaccination, necessary step for the development of a suitable vaccine for a possible clinical application (Nigro EA et al. 2009).

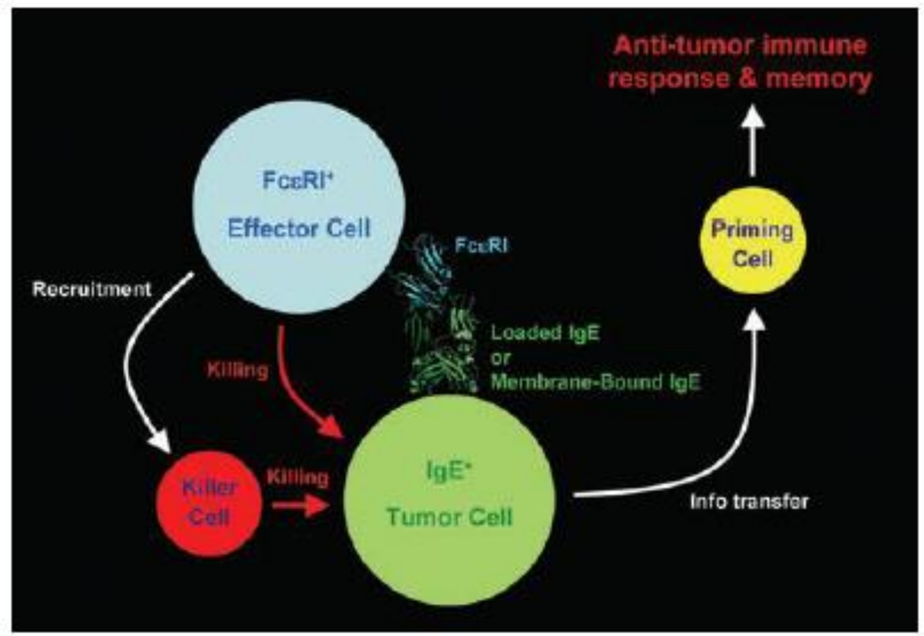


Figure 8. Representation of IgE-mediated mechanism in the anti-tumor immunotherapy.

VACCINIA VIRUS

Vaccinia virus (VV) is a virus belonging to the genus Orthopoxvirus of *poxviriade* family, and presents a linear genome with a double-stranded DNA and performs its replication cycle at the cytoplasm level of the host. VV morphologically is not different from other viruses of the same genus, such as variola virus, and is characterized by cross-reactivity to antigenic level, in fact, the infection by a member of the same family, confers protection against subsequent infections by all the others.

Although it is not known any natural host of VV and its exact origin remains unknown, its molecular biology has become the subject of numerous study, since it is a particularly flexible instrument for laboratory use, especially as an expression vector (Moss B 1996). The poxvirus represents a big target for immune system, although encoding a large repertoire of defense molecules versus host, and these molecules are non-essential for virus replication in culture. Some of these molecules are secreted by infected cells and prevent the activation of complement or the binding of interferons, interleukins or of TNF (Tumor Necrosis Factor). Other regulatory proteins, instead, remain within the cells preventing the action of interferon, induction of apoptosis, and antigen presentation (Smith GL et al. 1997).

MVA, an attenuated and non-replicative vector

The Vaccinia Virus is an infective virus whose use is subjected to special rules and restrictions, including the use of containment equipment with sterile rooms of microbiological safety and vaccination of laboratory staff. Two highly attenuated strains of VV, the NYVAC and Modified Virus Ankara (MVA), were approved by the intramural biosafety committee Institute of Health American (*National Institute of Health*, NIH) for the use in absence of microbiological safety room and also by non-vaccinated staff. The NYVAC virus was realized through selective deletion of eighteen genes, some of which are involved in viral tropism or in pathogenicity; in human cell lines, the replication of NYVAC virus is blocked at an early stage but a productive infection can be observed in African green monkey kidney cells (VERO) and in chicken embryo fibroblasts (CEF) (Tartaglia J et al. 1992).

Differently, MVA virus is blocked at the level of virion assembly, allowing the expression of heterologous proteins even in non-permissive cells. MVA virus grows to high titers in CEF cells and in a cell line derived from baby hamster kidney (BHK-21), but does not replicate in human cell lines and in other mammals due to multiple gene defects (Mayr A *et al.* 1975). The defects of MVA, accumulated after 570 serial passages in CEF, consist of six genomic deletions that determine a more highly attenuated phenotype and a considerable host range restriction (Meyer H et al. 1991).

rMVA, a good tool as vaccine against cancer

For the experimental therapy against tumors, cancers associated to viruses appear to be good targets for vaccines based on MVA; *in vitro* studies were performed for nasopharyngeal carcinoma associated with Epstein-Barr (Taylor GS et al 2004), and in a phase II clinical study it was demonstrated the efficiency of a MVA vaccine expressing the E2 antigen of the human papilloma virus (HPV) against cervical cancer associated with HPV (Corona Gutierrez CM et al. 2002). Vaccines based on recombinant MVA (rMVA) expressing different tumor-associated antigens specific for different types of cancer are in preclinical studies in murine models (Mulryan K et al. 2002; Hodge JW et al. 2003; Espenschied J et al. 2003). A rMVA expressing the MUC1 antigen is also in phase I clinical trial in patients with advanced cancer MUC1positive (Rochlitz C et al. 2003). Often these vaccines are combined with cytokines such as IL-2 (Rochlitz C et al. 2003) or costimulatory molecules which act as adjuvants.

Data from a clinical phase I study for the treatment of metastatic melanoma, indicate that vaccination with dendritic cells transduced with MVA (in patients who have the HLA-A*0201 allele locus in the MHC class I) can activate, *in vitro*, T cells response against both the recombinant antigen of tyrosinase, either against an epitope of the antigen H3 of VV's envelop (Drexler I et al*.* 2003, Di Nicola M et al*.* 2004).

Based on prior results, our group developed a novel protocol based on membrane IgE for anti-tumor vaccination to eliminate any possible problem of anaphylactogenicity caused by circulating IgE (Nigro EA et al. 2012).

The construction of a rMVA capable of expressing truncated human membrane IgE (rMVA-tmIgE) to be used in anti-tumor vaccination protocols, was generated using an innovative engineering method based on an extremely rapid selection procedure and color-driven (*red to green gene swapping*) (Di Lullo G et al. 2009; Di Lullo G et al. 2010)*.*

rMVA-tmIgE, able to induce the expression of the Cε3Cε4 portion of a human chimeric membrane IgE on the surface of cancer cells may be the best solution between efficacy and safety.

The inability of MVA to replicate in infected cells and therefore infect host surrounding cells, ensures that the expression of tmIgE-Cε3Cε4 remains confined to the tumor site.

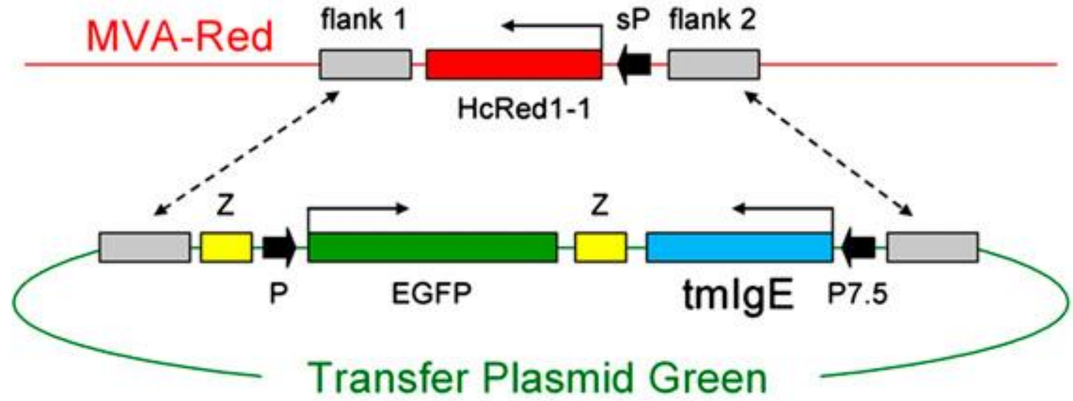


Figure 9. Genetic scheme of the infection/transfection reagents for the Red-to-Green technology.

rMVA-tmIgE was used to infect TS/A-LACK tumor cells *in vitro*, resulting in transport of tmIgE on the surface of infected cells. Human FcεRIα ($Fc\varepsilon RI\alpha^{-/-}hFc\varepsilon RI\alpha^{+}$) transgenic mice were vaccinated

subcutaneously with infected TS/A-LACK tumor cells and after fifteen days they were challenged with live TS/A-LACK cells. Mice immunized with rMVA-tmIgE-infected TS/A-LACK cells showed significant attenuation of tumor growth compared to mice immunized with control vaccine not expressing tmIgE. This second study showed again the role of FcεRI in IgE-active anti-tumor immunotherapy (Nigro EA et al. 2012).

Another interesting point in the use of MVA is given by several studies that demonstrated, in murine models, the efficacy of intratumoral treatment with MVA, resulting in reduction of the tumor mass and in increase insurvival of treated mice (Nemeckova S et al. 2007; Erbs P et al. 2008). Intratumoral treatment with viral vectors allows the infection of only resident cells inside the tumor mass thus ensuring a lower dispersion of viral vector resulting in lower toxicity to systemic level and in the increase of the local effect (Luo D and Saltzman WM 2000; Paielli DL et al. 2000).

For example, in an experimental study, C57BL/6 were challenged subcutaneously with TC-1/A9 cells, characterized by the expression of HPV 16 oncogenes and down regulation of H-2b molecules. At day 7 and 14 after inoculation of tumor cells, the mice were treated subcutaneously with highly immunogenic E7GGG.GUS DNA vaccine expressing the fused gene of modified HPV16 E7 (E7GGG) with *E.coli* β-glucuronidase; then, the tumor mass (when became palpable) was treated at intratumoral level with two doses (10^6pfu) of recombinant vaccinia virus MVA expressing the gene for murine granulocyte-macrophage colony-stimulating factor (MVA-GM-CSF). Data obtained have demonstrated a synergic effect given by the combined treatment of DNA vaccine with intratumoral injection of MVA-GM-CSF (Nemeckova S et al. 2007). Indeed, in mice also treated with MVA-GM-CSF compared to those treated only with DNA, it was observed a significant reduction of the tumor mass volume. Furthermore, it was shown that the local injection of MVA-GM-CSF induces the level of CD3+ T cells at the tumor mass level and this indicates that intratumoral delivery of MVA-GM-CSF may modify the microenvironment of the tumor, making it more accessible to the tumor effector T cells (NemeckovaS et al. 2007).

MATERIALS AND METHODS

Mice

In vivo experiments were conducted on randomized female and male mice (2–4 months of age) and performed in accordance with the Italian State and European regulations governing the care and treatment of laboratory animals (permission No. 04/2012 and 1193/2015-PR). Transgenic mice were bred in a conventional animal facility.
All mice enrolled in this project were housed with light/dark cycles of 12 hours, temperature of 22±2 °C, humidity of 55±10%, food and water ad libitum.

BALB/c Wild Type: BALB/c mice (female 2 months of age) were purchased from Harlan Laboratories.

IgE-KO: females and males (BALB/c *background*) kindly provided by Dr. Achatz. This transgenic mouse strain, which leads mutation at the level of specific domains in the ε germline gene (ΔM1ΔM2), was generated using the technique of gene targeting in embryonic stem cells. These mutations result in the absence of the transmembrane and cytoplasmic domains of IgE; moreover, their serum IgE levels are reduced of about 94-98% due to a smaller number of IgE-secreting plasma cells (Achatz G et al. 1997).

KN1: females and males (BALB/c *background*) kindly provided by Dr. Achatz. This transgenic strainexpresses a chimeric ε-γ1BCR, consisting of the extracellular domain of the ε gene and the transmembrane and cytoplasmic domains of the γ1 gene. In these mice the serum IgE levels are increased by 6 times, resulting from an elevated number of IgE-secreting cells (Achatz-

Straussberger G et al. 2008). The genotype of KN1 will be indicated as "**RRHH**" since they express the IgE-receptor FcεRI (R) and produce high levels of IgE (H) after antigen stimulation.

FcεRIα-KO: females and males (BALB/c *background*) kindly provided by Dr. Dombrowicz. This transgenic mouse strain was generated by homologous recombination in embryonic stem cells, using a plasmid designed to inactivate the murine α chain of the FcεRI receptor. These mice express a murine FcεRI receptor lacking the α chain and consequently unable to bind IgE, therefore non-functional (Dombrowicz D et al. 1993). For convenience the genotype of these mice will be indicated as "**rrhh**" since they lostthe α chain of the FcεRI receptor "r" and express normal levels of IgE (h).

KN1/FcεRIα-KO (also called as double mutant, DM): females and males produced by Dr. Vijay Yenagi at our animal facility. This transgenic mouse strain was obtained by a cross of KN1 (RRHH) males with FcεRIα-KO (rrhh) females. This murine model is characterized by increased serum levels of IgE and by the loss of FcεRIα (Nigro EA et al. 2016).Therefore the genotype of these mice will be indicated as "**rrHH**".

Genotyping

To characterize transgenic mice, DNA was extracted by tail biopsy and tested by PCR (Polymerase Chain Reaction). Briefly, tail biopsy of each animal (> 21 days of age) were lysed in 0.5 ml of Lysis buffer (1% SDS, 400mM NaCl, 5mM EDTA [pH 8.0], 100mM Tris [pH 8.0]) containing 0.2 mg/ml of Proteinase K. Samples were incubated overnight at 56°C and, following phenol/chloroform extraction, resulting DNA was precipitated in 100% ethanol, washed with ice-cold 70% ethanol and finally resuspended in MilliQ water. DNA purity was assessed by reading the absorbance at 260 nm and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific); a ratio of ~1.8 is generally accepted as "pure" for DNA. Subsequently, 300-400 ng of DNA were amplified using Taq DNA Polymerase (Invitrogen). PCR reaction was performed as follows: denaturing at 94 °C for 5 minutes, amplification for 30 cycles (denaturation for 1minute at 94 °C, annealing for 1 minute at 60 °Cand extension for 1 minute at 72 °C) followed by 7 minutes at 72 °C.

To characterize transgenic mice we used different primers. The physiological expression of IgE, characterized by the presence of "h" allele, was verified using the following primers:

h-forward:5'GAAATGGACCTATAAGCTTAGAGCCTTCC3'

h-reverse:5’GATGTTCTTCTAAGCTTTGTCTCAAAG3’ that produced DNA amplicons of 930 bp.

High levels of serum IgE, characterized by the presence of “H” allele, were verified using the following primers:

H-forward: 5’GAATGGGCTGACCGCTTCCTC3’

H-reverse: 5’GGTAAAGGCTGCTTCCTAGTC 3’ that produced amplicons DNA of 1.2 Kb.

The normal expression of murine FcεRIα chain, characterized by “R” allele, was tested by:

R-forward: 5’TTGGCTGCTCCTTCAGACAT3’

R-reverse:5’CTCTCTAATGGAGACGGGGC3’ that produced amplicons DNA of 161bp.

Finally, the loss of the FcεRIα chain in mice was tested by:

r-forward:5’CTTGGGTGGAGAGGCTATTC3’

r-reverse: 5’CTCTCTAATGGAGACGGGGC3’that produced amplicons DNA of 1.3 Kb.

The PCR products were analysed by fractionation in a 1.2% agarose gel and visualized with ethidium bromide staining. The images were captured using Syngene GBox-HR Gel Doc System.

Cell lines

TS/A: mammary adenocarcinoma cell line of BALB/c origin, maintained in RPMI-1640(GIBCO), 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma-Aldrich) (Benigni F et al. 2005).

N2C**:** HER-2/neu expressing N2C primary mammary carcinoma cell line derived from female BALB-neuT mice. These cells were maintained in DMEM supplemented with 10% of FBS, 100 U/ml penicillinand,100 U/ml of streptomycin.

RBL-2H3: rat basophilic leukemia cell line (Kulczycki Jr A and Metzger H 1974) maintained in DMEMsupplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin.

CEF: primary culture of chick embryo fibroblasts, kindly provided by Dr. Soprana and Dr.Panigada; CEF are maintained in DMEM supplemented with 5% of FBS, 1% of chicken serum (CS) and 1% of penicillin/streptomycin.

NS1 myeloma cells: murine myeloma cells, kindly provided by Dr. Soprana; these cells were maintained in RPMI-FCS (RPMI supplemented with 100 U/ml of Penicillin, 100 μg/ml of Streptomicin, 0.25μg/ml of Amphotericin β, 2mM of Glutamine and 10% of FCS).

BHK-21: cell line of hamster kidney (baby hamster kidney) (Macpherson I and Stoker M 1962) maintained in RPMI (GIBCO) supplemented with10% bovine serum (FCS) (Euroclone Ltd.) and 1% penicillin / streptomycin(GIBCO).

All cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2.

Flow cytometry

-Characterization of N2C cells for Her-2/neu expression: to characterize N2C cells, $5x10^5$ cells were washed in PBS and incubated for 1 hour at 4°C with anti c-Erb B2/c-Neu [Ab4], 1:40 in PBS-5% BSA (bovine serum albumin); subsequently, cells were washed and incubated for 1 hour at 4°C with anti-mouse IgG-biot (Sigma), 1:100 in PBS-5% BSA. Specific binding was revealed by incubation with Streptavidin-PE (Pharmingen), 1:300 in PBS-5% BSA for 30 minutes at 4°C, followed by three washes.

-Evaluation of $CD8^+$ T cells depletion: CD8+ T cells depletion was assessed by cytofluorimetric analysis on peripheral blood. Briefly, 200μl of peripheral blood were collected by venipuncture, at tail level, in heparinised tubes; then, the samples were incubated with 2 ml of ACK Lysis buffer (150 mMNH_4Cl, 10mM $KHCO_2$, 0.1 mMNa_2EDTA, in sterile water) for 2 minutes to eliminate red blood cells. After washing, cells were incubated with rat anti-mouse CD8a-Cy5 (clone 53-6.7, BD Biosciences), 1:100 in PBS-5%BSA for 60 minutes at 4°C, followed by three washes.

-Evaluation of TS/A cells infection: cells, infected with rMVA-tmIgE (Nigro EA et al. 2012), were incubated with mouse anti-human IgE-biot (BD Pharmingen), 5 μg/mL in PBS-5% BSA,for 30 minutes at 4°C. After 2 washes with PBS, specific binding was revealed through Streptavidin-PE (Pharmingen), 1:300 in PBS-5% BSA for 30 minutes at 4°C.

All samples were resuspended in 500 µl of PBS and acquired by flow cytometry (FACSCalibur, Becton Dickinson, USA) and analysed by CellQuest Pro software.

Implantation of Tumor cells in mice

-Immunization and implant of TS/A tumor cells: five mice for each group were immunized by subcutaneous (s.c.) injection of 10^5 irradiated (10,000 rad) TS/A tumor cells per animal, at the tail base level. 14 days after immunization, mice were s.c. challenged in the abdomen with $2x10^5$of live TS/A tumor cells resuspended in 200µl of PBS, using insulin syringe (gauge 29G).

-Implant of N2C tumor cells by subcutaneous administration: five mice for each group were challenged with $6x10^5$of N2C cells per animal; tumor cells were resuspended in 200µl of PBS and s.c. inoculated in the abdomen, using insulin syringe (gauge 29G).

-Implant of N2C tumor cells by intravenous (i.v.) administration: five mice for each group were challenged with $1.2x10^6$of N2C tumor cells per animal; tumor cells were resuspended in 200µl of PBS containing 2.5% of heparin and then were inoculated i.v.in the lateral tail vein using insulin syringe (gauge 29G).

Tumor growth monitoring

Tumor growth was monitored every two days by measurement of tumor volume using an electronic digital caliper. Tumor volume, V, was calculated using the formula: $V = 4/3 \times \pi \times (d_1/2 \times d_2/2 \times r)$, in which d_1 is the horizontal diameter, d_2 is the vertical diameter, and r is the thickness of tumor protruding from the surface of normal skin. When exceeding 500 mm^3 of tumor volume, animals were euthanized by cervical dislocation.

Histology

-Evaluation of tumor growth: to evaluate s.c. N2C tumor growth at different times, labeled injection site or tumor mass were harvested at 6, 24 hours and 13 days, then fixed in buffered 4% formalin and embedded in paraffin. Multiple 3µm sections from paraffin-embedded samples were cut and stained with hematoxylin and eosin for microscopic analysis.

-Evaluation of metastases: 35 days post i.v. injection of N2C tumor cells, lungs were analysed for metastases using the following procedure: a mid-line incision from below the diaphragm to above the throat was used to expose the trachea before opening the chest cavity. Subsequently the trachea was cut at the top and the lung was extracted from the thoracic cage. Approximately 2 mL of a solution of 15% (v/v) Indian ink in water was infused into the lungs by inserting a cannula into the trachea and then placed in sterile water for at least 5 minutes to remove excess ink. Thereafter, lungs were placed in Fekete solution (70% [v/v] ethanol, 15% [v/v] sterile water, 10% [v/v] formaldehyde and 5% [v/v] of glacial acid) for 48 hours, then tumor masses were visible as white nodules on a black normal lung tissue surface (Wexler H 1966). Moreover, samples were embedded in paraffin and multiple 3µm sections were cut and stained with hematoxylin and eosin for microscopic analysis.

In both cases pictures were taken using the AxioCam HRc with the AxioVisionVs 40 V 4.6.3.0 (Zeiss).

Mouse serum collection

Approximately 500µl of peripheral blood were collected by tail vein puncture and incubated for 30 minutes at room temperature (RT) to allow clotting. Subsequently, serum was obtained after two steps of centrifugation at 500g and 2,000g for 10 minutes and stored in a sterile tube at 4°C before use.

Mediator release assay

RBL-2H3 cells expressing the murine FcεRI receptor were seeded and let adhere overnight in96-well plates in DMEM 10% FBS (8x10^4 cells / well). The following day, cells were incubated in DMEM with 100 ng of mouse IgE k isotype control Ab (clone C38-2; BD Biosciences) for 2 hours at 37°C.Cells were then washed with PBS and incubated in triggering buffer (119 mMNaCl,5 mMKCl, 26 mM PIPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$,0.1% BSA [pH 7.2]) with 100 ngof anti-mouse IgEmAb (R35-72) for 1 hour at37°C (represented positive control). Alternatively, 2x$10^5$of N2C tumor cells pre-incubated with serum from mice challenged with tumor cells (or supernatants derived hybridomas in case of specific-IgE isolation experiment), were washed, resuspended in

triggering buffer, and added to RBL-2H3 cells. Plates were centrifuged for 5 minutes at 300 g and incubated for 1 hour at37°C. The release of β-hexosaminidase by RBL-2H3 cells was detected in the culture supernatant. In detail, supernatants were transferred to a new plate and 1 mM of p-nitrophenyl-N-acetyl-β-D-glucosamide (Sigma-Aldrich) 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 0.1 M stop solution (0.1 MNa_2CO_3, 0.1 M $NaHCO_3$, [pH 10.0]), and the absorbance was read at 405nm (Vangelista L et al. 2005). Results are calculated as percentage of the positive control (IgE plus crosslinker).

Isolation of mice tumor specific-IgE

10 days before the fusion, NS1 myeloma cells (murine myeloma) were thawed and cultured in RPMI-FCS (RPMI supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomicin, 0.25µg/ml of amphotericin β, 2mM of glutamine and 10% of FCS); the cells were expanded to obtain a minimum amount of $50x10^6$. Ten days before the fusion, the cells were passed 1:2 with fresh medium to obtain a population with an exponential growth; in addition, one day before the fusion cells were diluted 1: 3 with fresh medium.

In order to isolate tumor specific-IgE, KN1 mice have been challenged intraperitoneal (i.p.) with three doses of $6x10^5$ N2C tumour cells at day -3, -2 and -1 prior to extract spleen and bone marrow cells to fuse with NS1 myeloma cells and produce hybridomas. To promote the fusion between splenocytes and bone marrow cells with NS1 myeloma cells, the cells were enriched *in vitro* with IL-6 or with splenocytes derived from Balb/c wild type mice. For this reason at day -2, also splenocytes from BALB/c were extracted and plated as feeder in 96-well plates (1 spleen/fusion). At day 0, KN1 mice were sacrificed and spleen and bone marrow were extracted to isolate cells. For spleen, cells were isolated by mechanical disintegration in RPMI P/S/A (RPMI supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomicin and, 0.25µg/ml of amphotericin β). Collected splenocytes were washed in the same medium and resuspended in 50ml of RPMI P/S/A. To isolate the cells from bone marrow, the femurs of KN1 mice were extracted, accessory muscles were eliminated and then both extremity of the femurs were cut using a scalpel. Following, 2ml of RPMI P/S/A was injected into one of the two extremities of the femur, using a 1ml insulin syringe with a 29G needle, in order to rinse the inside of the bone marrow and extract the contained cells.

In parallel, myeloma cells were observed using an optical microscope, in order to choose the most viable cells and collect them in a 50 ml tube; it was important, in fact, that fusion was performed using cells in exponential growth phase and in maximum vitality condition. At this point splenocytes, bone marrow cells and NS1 myeloma cells were each in a 50 ml tube; after centrifuging at 1300 rpm for 10 minutes at 4 °C and cell counting, splenocytes and bone marrow cells have been mixed. Subsequently, NS1 myeloma cells were mixed with splenocytes/bone marrow cells in the ratio 1: 4 (1 NS1 myeloma cell : 4 splenocytes/bone marrow cells) in a special culture medium RPMI-HAT (RPMI supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomicin, 0.25µg/ml of amphotericin β, 2mM of glutamine, 20% of FCS, 1% of HAT 50x [hypoxanthine-aminopterin-thymidine; Bio-SPA cod. F0483] and, 4% of Briclone [BricloneHybridoma Coning Medium sterile; CABRU cod. 08BRI]).
The cell suspension obtained was distributed into ten 96-well plates (200µl/well) using a multichannel; cells were incubated for 10-15 days at 37 °C and 5% of CO_2.At the end of selection in RPMI-HAT medium, the supernatants of the wells with cell growth were analyzed for the presence of IgE by ELISA test and positive clones for IgE were evaluated for their tumor-specificity through mediator release assay.

ELISA test for IgE clones

In order to evaluated the isolation of IgE clones, a 96-well plate was coated with 2,5 µg/ml of anti-IgE in PBS (R35-72) overnight and the next day, the plate was blocked with PBS-1% BSA for 2 hours at RT. After two washes with PBS-0.05% Tween, supernatants of clones or IgE standard (starting from 500ng/ml to 31.3 ng/ml) were incubated in 100µl/well of RPMI-20% FBS at 4°C, overnight. Subsequently, after 4 washes with PBS-0.05% Tween, 2µg/ml of anti-IgE biot (R35-118) in PBS-0.1% BSA was added to plate, for 1.5 hours at RT. Later, the plate was incubated with streptavidin-HRP (1:1000 in PBS-0.1% BSA), 100µl/well for 1 hour at RT and, after four washes, 100µl/well of OPD substrate (OPD, o-phenylenediaminedihydrochloride; Thermo Fisher Scientific) in dark was added. The reaction was stopped with 100µl/well of H_2SO_4 2N and the absorbance was read at 490nm.

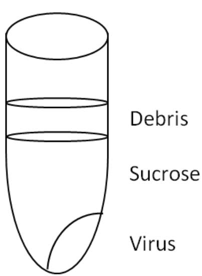
In vivo CD8^{+} T cells depletion

KN1 mice were depleted of CD8^{+} T cells by i.v. injections with 300 µg of rat anti-mouse CD8α ascite (clone 53-6.7, Abnova) at day 6, 3 and 1 before tumor challenge. Subsequently, CD8^{+}T cells depletion was monitored by cytofluorimetric analysis on peripheral blood (as described in the previous paragraphs).

Virus Amplification

For MVA-HcRED or rMVA-tmIgE amplification, an aliquot of each virus was kindly provided by Dr. Soprana and Dr. Panigada. MVA-HcRED is a Modified Vaccinia Ankara characterized by the presence of the gene for the red fluorescent protein expression HcRED (di Lullo G. et al. 2009). rMVA-tmIgE, instead, is a recombinant Modified Vaccinia Ankara characterized by the ability to express a truncated version of human mIgE, capable to bind and activate FcεRI receptor (Nigro EA et al. 2012).

For the virus amplification we adopted the following protocol: to obtain a high number of virus copies (stock), CEF cells seeded on flasks at 80% of confluence, were infected with 50µl of concentrated virus. After 4-7 days, virus effective replication was evaluated through CEF lysis and then, cells were scraped and transferred into 50 ml falcon/Flask and stored at -80°C. Subsequently, samples were thawed and distributed into Nalgene tubes and spun for 1 hour at 24000 g at4°C; resulted pellets were resuspended in 1ml of 10mMTris pH 9 per tube, then subjected to three cycles of freezing, thawing, vortexed and sonicated and finally spun for 5 minutes at 1800 g at4°C. Resulting supernatants were collected and the remained pellets were resuspended in 1ml of 10mMTris pH 9 and then, the previous steps were repeated. Supernatants obtained were collected and pooled together. Pooled supernatant was diluted up to 15 ml with 10mM of Tris pH 9, added on the top layer of 15 ml 36% sucrose in Tris pH 9; this step was followed by a centrifugation for 1 hour at 30000 g at 4°C and in order to obtain:



After discarding the supernatant, the final pellet containing the virus was resuspended in 1 ml of formulation buffer (10mM NaCl + 280mM Tris pH 7.7 [1:1, v:v]) and stored at -80°C before virus titration.

Virus titration

The assessment of viral titer was performed on BHK-21 (baby hamster kidney) cells through the method of the terminal dilution. Cells (2000/well) were seeded in 96-well plates and let adhere for at least 4 hours. For each titrated stock, serial dilutions in powers of 10 (from 10^{-2} to 10^{-12}) were prepared and seeded in a known volume (50ul) in 16 wells/dilution.

In the case of viruses expressing HcRED, the detection of positive wells for the infection was carried out 3-5 days after seeding by the observation of plate with fluorescence microscopy. In the case of viruses lacking the fluorescence cassette, a preliminary screening was carried out to identify cytopathic effect 3-5 days after seeding, to allow virus expansion; subsequently, the titration plates are replicated on a same number of plates containing fresh BHK-21, and at this stage, the more concentrated virus within each well, results in obvious foci of lysis allowing the reliable identification of positive wells.

Replica plates were then fixed for 10minutes with methanol : acetone (1:1, v:v), washed two times with water and incubated 5 minutes with crystal violet 0.5% in water followed byseveral washes to remove the excess of colorant. The presence of lysis foci was identified by the absence of purple staining, since the suffering infected cells have been detached during fixation with methanol : acetone. After counting the positive wells for each dilution, it is possible to calculate the virus titer based on the Poisson distribution: $m = -\ln(f)$ where m indicates the average number of infectious viruses per well, and f is the fraction of non-infected replica. The result is expressed as pfu/ml (plaque forming units per ml).

In vitro infection of tumor cells

To test the good infection of tumor cells by MVA-HcRED virus, TS/A cells were seeded in 6-wellplate ($2x10^5$ cells/well) and infected at 1, 5 or 10 MOI (multiplicity of infection) with virus. After 2 hours of infection, cells were washed with medium to remove excess virus and after

24hours the infection success was assayed using fluorescence microscopy through the identification of cells emitting red fluorescence.

Intratumoral tumor mass treatment

Mice received a dose of $2x10^5$ tumor cells (TS/A) injected s.c.in the abdomen. When the tumor reached 100-200mm^3, viruses wereinjected (10^7pfuof MVA-HcRED or MVA-tmIgE, in 100µl of PBS) into tumor mass, using an insulin syringe with a 30 gauge needle.

After24 and 48 hours from treatment, the infection of cells into tumor mass was evaluated through immunohistochemistry assay.

Immunohistochemistry

Tumor masses deriving from 2 mice per group were collected at 24 and 48 hours after intratumoral treatment. Samples were fixed overnight in 10% buffered formalin (Sigma-Aldrich) at RT and then moved in 70% ethanol until processing.

After an abundant washing in water, samples were processed for paraffin embedding through a graded ethanol series using STP 120 Spin Tissue Processor (Bio Optica). Paraffin blocks were sectioned at 2-3µm for histological and immunohistochemical (IHC) examination, and sections were attached to glass slides coatedwith poly-L-lysine. Slides were dried overnight at 37°C to optimize sample adhesion. Sections were then deparaffinized with xylene and tissues were rehydrated in sequentially graduated ethyl alcohol.

To test the integrity of samples after processing, all tissues were stained with hematoxylin and eosin (H&E) and examined microscopically (images acquired by Olympus BX51, Japan) before IHC examination.

For IHC test, slides were incubated in Sodium Citrate buffer (BioOptica #15-M103) at 90°c for 20 minutes and after two washes with dH2O, slides were incubated in H_2O_2 0,3% in TBS (Tris base 50 mM, NaCl 150 mM in dH2O at pH 7.5) for 15 minutes to reduce nonspecific background staining due to endogenous peroxidase.

The sections were washed twice in TBS + 0.025% of Triton-X before the application of Blocking buffer (5% of total serum, 5% of BSA in TBS) for 1-2 hours at RT.

To detected tumor cellinfection by MVA-HcRED, 20-50 µl of primary antibody (rabbit anti-vaccinia virus, Quartett) in Diluent buffer (1% of BSA in TBS) was applied on the slides; to detected the

infection by rMVA-tmIgE, 20-50 µl of goat anti-human IgE-HRP conjugated (KPL) were applied on the slides. Samples were then incubated overnight at 4 °C. After being washed three times in TBS + 0.025% of Triton-X, 20-50 µl of goat anti-rabbit IgG-HRP (Santa Cruz) was applied on each slide and samples were incubated for 1 hour at RT.

Finally, after three washes, tissues were further incubated for 1-5 minutes at RT in a solution of DAB chromogen (3,3'-diaminobenzidine), then tissues were counterstained with Mayer's haematoxylin, washed in water, and coverslips were applied with mounting media. To exclude any aspecific binding of the secondary antibody, some sections were not incubated with primary antibody and were used as negative controls.

RESULTS

Effect of endogenous IgE in tumor growth

To evaluate a possible role of endogenous IgE in cancer immunosurveillance, we tested the ability of TS/A tumor cells to grow in mice engineered to modify their IgE system. For this first experiment KN1, IgE-KO and wild type (WT) mice were enrolled; in KN1 mice the serum IgE levels are increased by 6 times, resulting from an elevated number of IgE-secreting cells, conversely, IgE-KO mice are characterized by the absence of circulating IgE.

For each group were enrolled 5 mice which were immunized (or not) by subcutaneous inoculation, at the tail level, of 10^5 TS/A irradiated cells. After 14 days, the mice were treated subcutaneously (in the right flank) with $2x10^5$ of TS/A live cells. Afterwards, when the tumor became palpable, about 10 days after inoculation, we started to monitor the growth of the tumor mass and the survival of mice enrolled in the experiment (Fig. 1-A and 1-B).

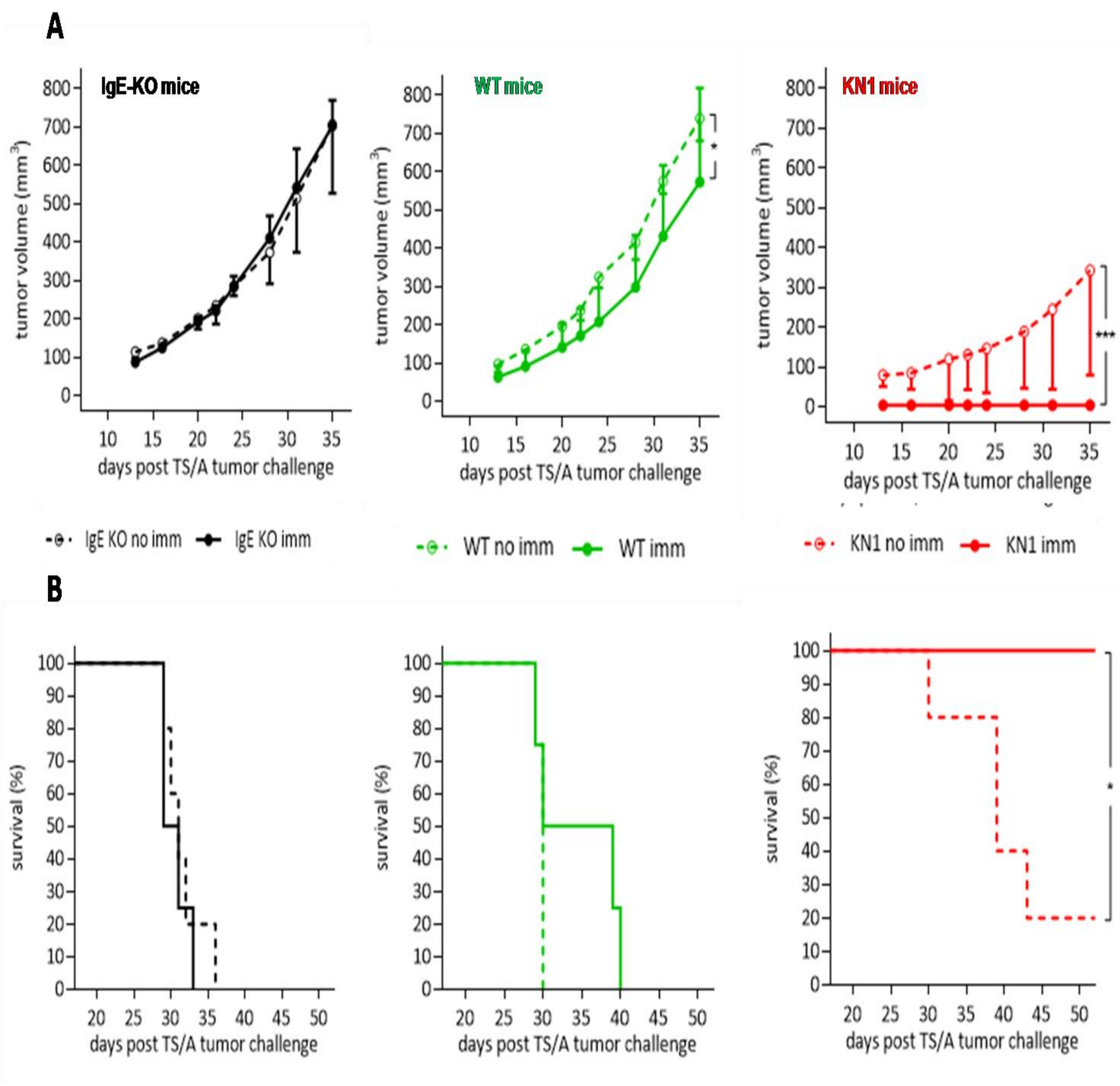


Figure 1. IgE-KO, WT and KN1 mice show different susceptibility to TS/A tumor growth. **A,** Tumor growth curves of IgE-KO (black lines), WT (green lines), and KN1 (red lines) mice challenged with live TS/A tumor cells. **B,** survival of IgE-KO, WT and KN1 mice with (solid lines) or without (dashed lines) prior immunization with irradiated TS/A cells as in A. Tumor volume growth curves were estimated by a non-linear mixed effect model and survivals by Kaplan-Meier analysis (log-rank test). The experiment shown indicates 3-4 replicates. *n*=5 mice. Error bars indicate SD. *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$.

As shown in Figure 1-A, the immunization with TS/A irradiated cells, prior to inoculation of tumor live cells, had no effect on tumor growth in IgE-KO mice. Conversely, in immunized WT mice, the tumor volume at 35 days post-TS/A challenge was significantly smaller, as compared to immunized WT mice (Fig. 1-A). Moreover, the growth of the tumor mass in unimmunized WT mice was equal to that of IgE-KO mice groups (Fig. 1-A).

In unimmunized KN1 mice, tumor growth was markedly lower than the other groups (IgE-KO and WT immunized and not), in fact, after 35 days from tumor implantation, the tumor mass volume had not even reached 400mm^3, widely exceeded in value in IgE-KO and WT groups. Surprisingly, in immunized KN1 group, 100% of animals were completely protected from tumor development (Fig. 1-A); in addition, immunized KN1mice that were subsequently challenged for a second time, never showed tumor growth, demonstrating an anti-tumor immunity persistence (data not shown).

The trend of tumor growth reflected exactly the trend of survival. Indeed, in IgE-KO mice, the survival was not affected by immunization, unlike WT group in which the immunization extended the survival of 10 days (Fig. 1-B). Moreover, in accordance with the reduced tumor growth, the survival of unimmunized KN1 mice was prolonged compared to the survival of WT and of IgE-KO mice, whereas 100% of survival was observed in immunized KN1 mice (Fig 1-B).

Characterization of N2C adenocarcinoma cells

Afterwards, we decided to extend our investigation usingN2C cancer cells, a primary mammary carcinoma cell line derived from BALB/NeuT and expressing the HER-2/neu receptor. We introduced this cell type in the study because these cells are less aggressive than the TS/A tumor cells: in fact no tumor growth is observed in KN1 mice, even without immunization.

Before their use, N2C cells were characterized for the expression of HER-2/neu through cytofluorimetric analysis as shown in figure 2.

To monitor the effective expression of HER2/neu receptor on cells, $5x10^5$of N2C tumor cells were incubated with anti-HER2/neu antibody and the signal of protein expression was detected by use of a biotinylated "second" antibody followed by streptavidin. As shown in the histogram of figure 2, the majority of cells expressed HER2/neu receptor (blue line). As a negative control, N2C cells were incubated alone (black line), or incubated with only streptavidin (green line) or with biotinylated anti-IgG antibody and streptavidin (red line).

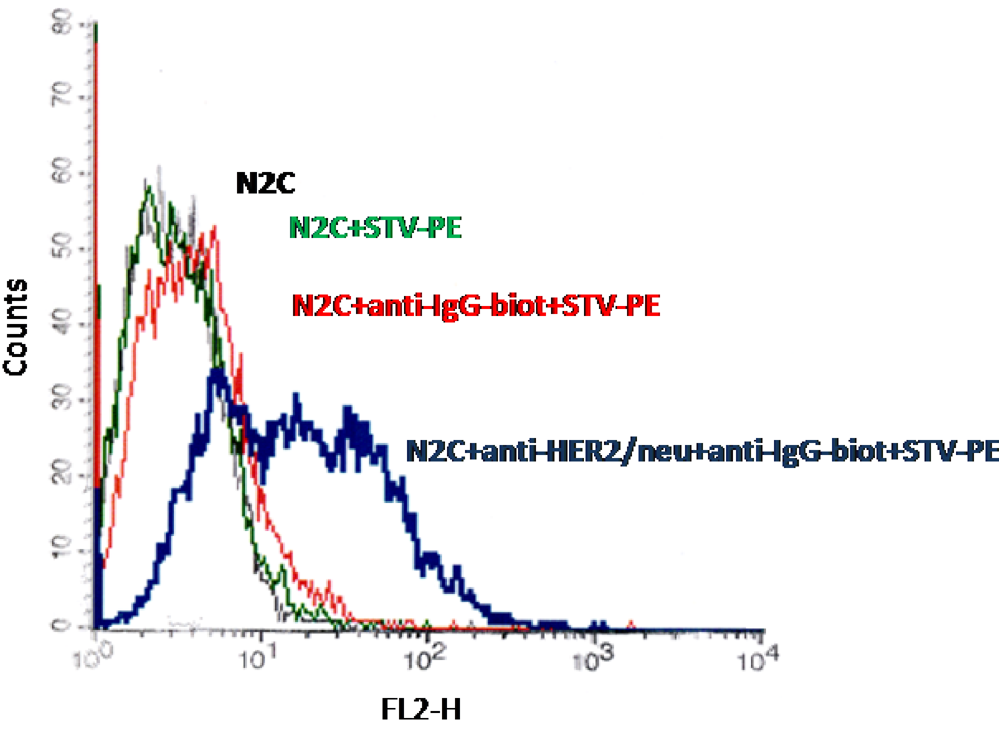


Figure 2. **FACS analysis of HER2/neu expression**. N2C tumor cells expressed HER-2/neu (blue line); unstained cells (black line); cells either incubated with streptavidin PE (green line) or with biotinylated anti-IgG antibody (red line) are also shown .

Tumor protection in KN1 mice without immunization

In the first *in vivo* experiment, we had evaluated the protection againsttumor developmentin KN1mice after TS/A tumor cells implantation. In this second experiment, we tested whether the antitumor protection was also evident using the tumor cell line N2C, characterized by slower growth, but high immunogenicity.

For this experiment were used KN1, IgE-KO and WT (5 mice for each group) mice. $6x10^5$of N2C tumor cells were inoculated, subcutaneously, in the right flank of mice and when the tumor became palpable, we started to monitor the growth of tumor mass and the survival of mice(Fig. 4 A and B).

Generally, tumor growth in IgEKO and WT groups was slower than that observed in the previous experiment using TS/A cells; indeed tumor volume never reached the value of 500 mm^3 (Fig. 3 A), greatly exceeded in the same mice of the first experiment.

In detail, N2C tumor growth, even without prior immunization, was significantly slower in WT mice compared to IgE-KO mice and in KN1 mice no tumor growth was ever observed (Fig. 3 A). Also in this case, survival reflected the trend of tumor mass growth. Indeed, as shown in figure 3 B, IgE-KO mice died 15 days earlier than WT, while KN1 mice showed 100% of survival (Fig. 3 B).

At this point we had two independent tumor models in which we observed the existence of an antitumor protection given by endogenous IgE.

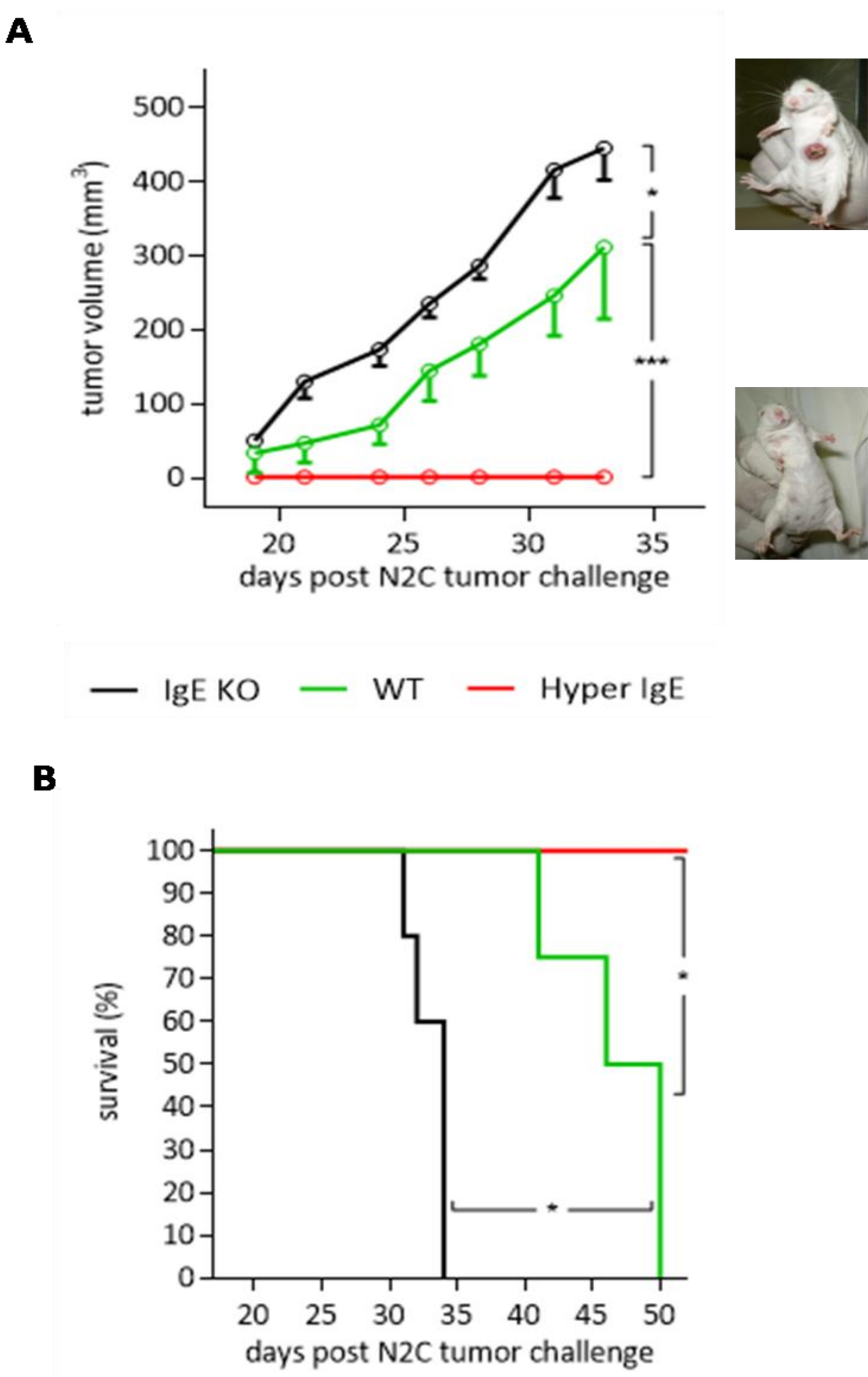


Figure 3. N2C tumor cells do not take root in KN1 mice. A, tumor growth curves in IgE-KO (black line), WT (green line), and KN1 (red line) mice challenged with live N2C tumor cells without prior immunization. Beside are shown representative images of tumor growth in IgE-KO and KN1 mice. **B,** survival of the same mice as in A. Tumor volume growth curves were estimated by a non-linear mixed effect model and survivals by Kaplan-Meier analysis (log-rank test). The experiment shown indicates 3-4 replicates. *n*=5 mice. Error bars indicate SD. *P< 0.05; **P< 0.01; ***P< 0.001.

To evaluate the engraftment of cancer cells in KN1mice, we performed histological analysis of the injected tumor cells area. In this experiment, KN1and WT mice that received $6x10^5$ N2C tumor cells, were sacrificed at different time points and the tumor cell implantation area was collected and histologically analyzed by hematoxylin-eosin.

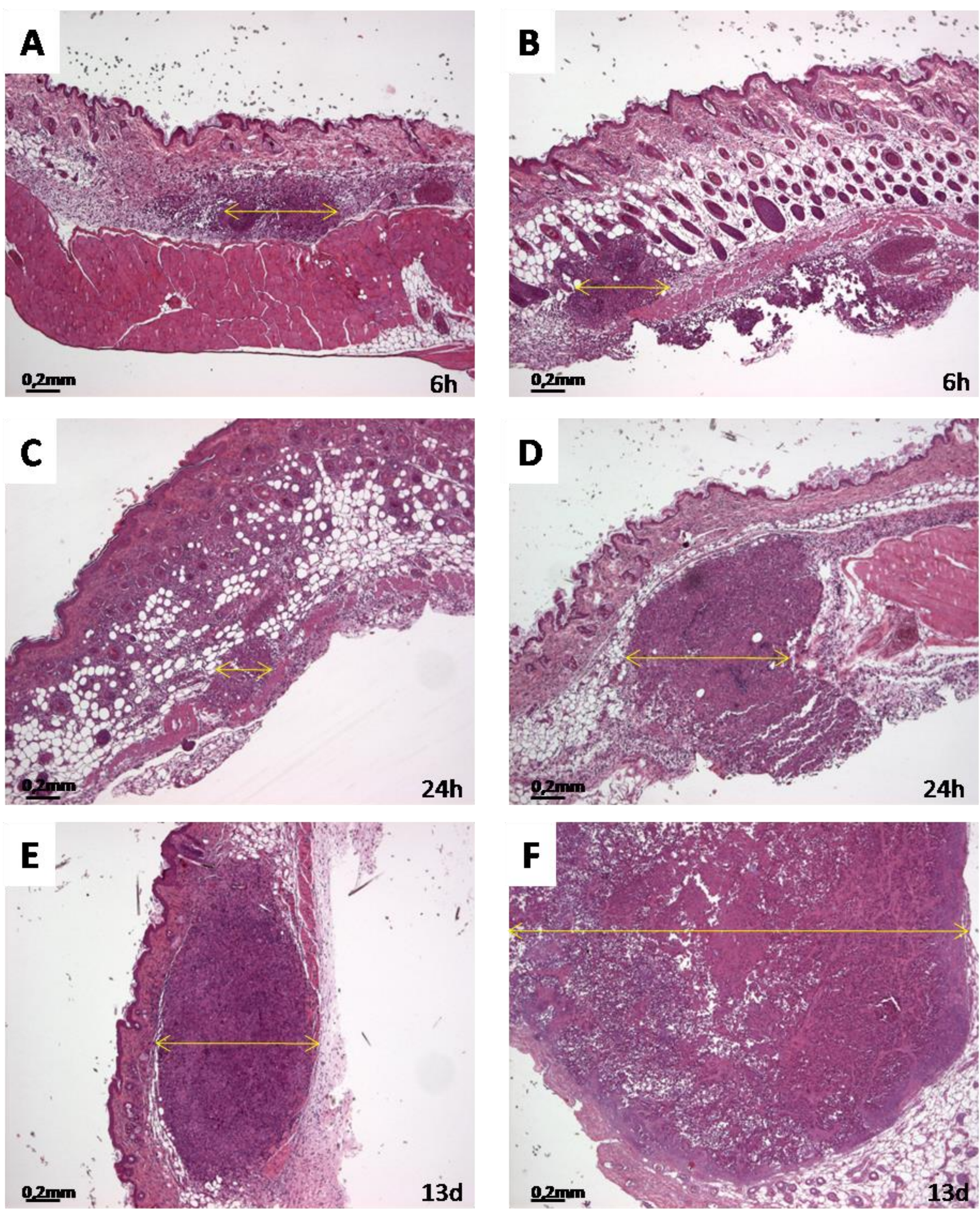


Figure 4. Kinetic of N2C cells engraftment in KN1 and WT mice. **A-F**, Hematoxylin & Eosin stained sections of N2C tumors at 6, 24 hours and 13 days after injection in KN1 mice (**A, C** and **E**) and in WT mice (**B, D** and **F**).

Histological analysis 6 hours after tumor cells implantation showed that cells engraft equally in both KN1 and in WT mice (Fig. 4 A-B); 24 hours later, unlike KN1 mice, in which the tumor has not yet grown compared to the previous time (6 hours), in WT mice the growth of tumor cells is evident (Fig. 4 C-D).

After 13 days post tumor cells implantation, when the mass became palpable, only small tumors were detected in KN1 mice, while the mass in WT mice were bigger (Fig. 4 E-F). At 30 days post inoculation, no tumors in KN1mice had grown, while the tumors in WT mice had grown enormously (data not shown).

Protection from lung metastases in KN1 mice

KN1 and WT mice were also treated with $1.2x10^6$ N2C cells by intravenous injection into the tail vein. After 35 days post treatment, mice were sacrificed, their lungs were extracted and stained through infusion of Indian Inkdye (infused into the lung via trachea) and finally fixed in Fekete solution. Lungs were analysed macroscopically through the observation of tumor foci on the external surface; then lungs were embedded in paraffin and multiple 3µm sections were cut and stained with haematoxylin and eosin for microscopic analysis.

As shown in figure 5, in KN1 mice no tumors were identified, unlike WT mice in which the tumor masses (represented by white patches) filled almost the whole external surface of the lungs (Fig. 4 A-B-C-D). Moreover, histological analysis showed that even within the lungs, KN1 mice were tumor free, unlike WT mice in which a large number of metastases were observed (Fig. 5 E-F).

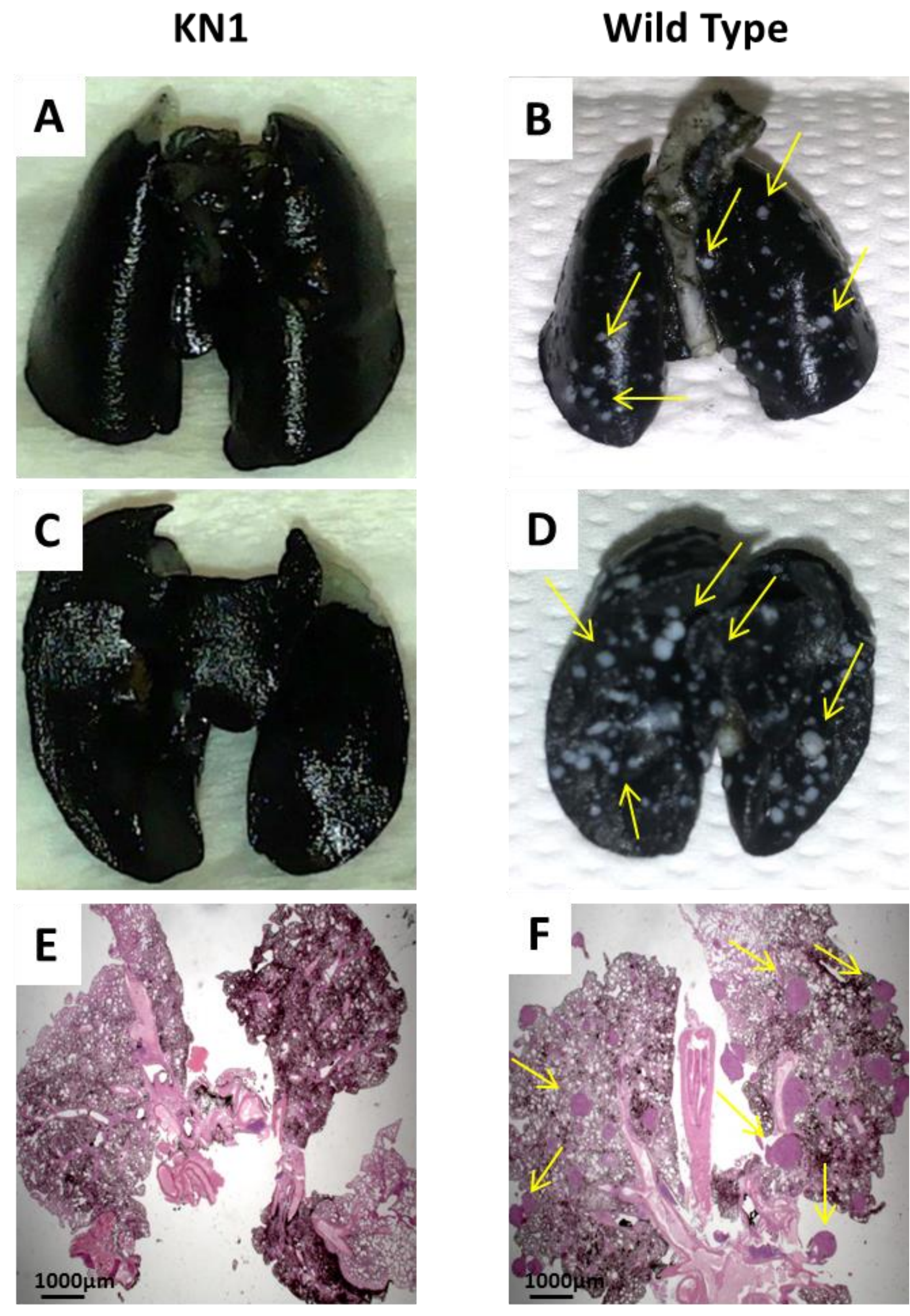


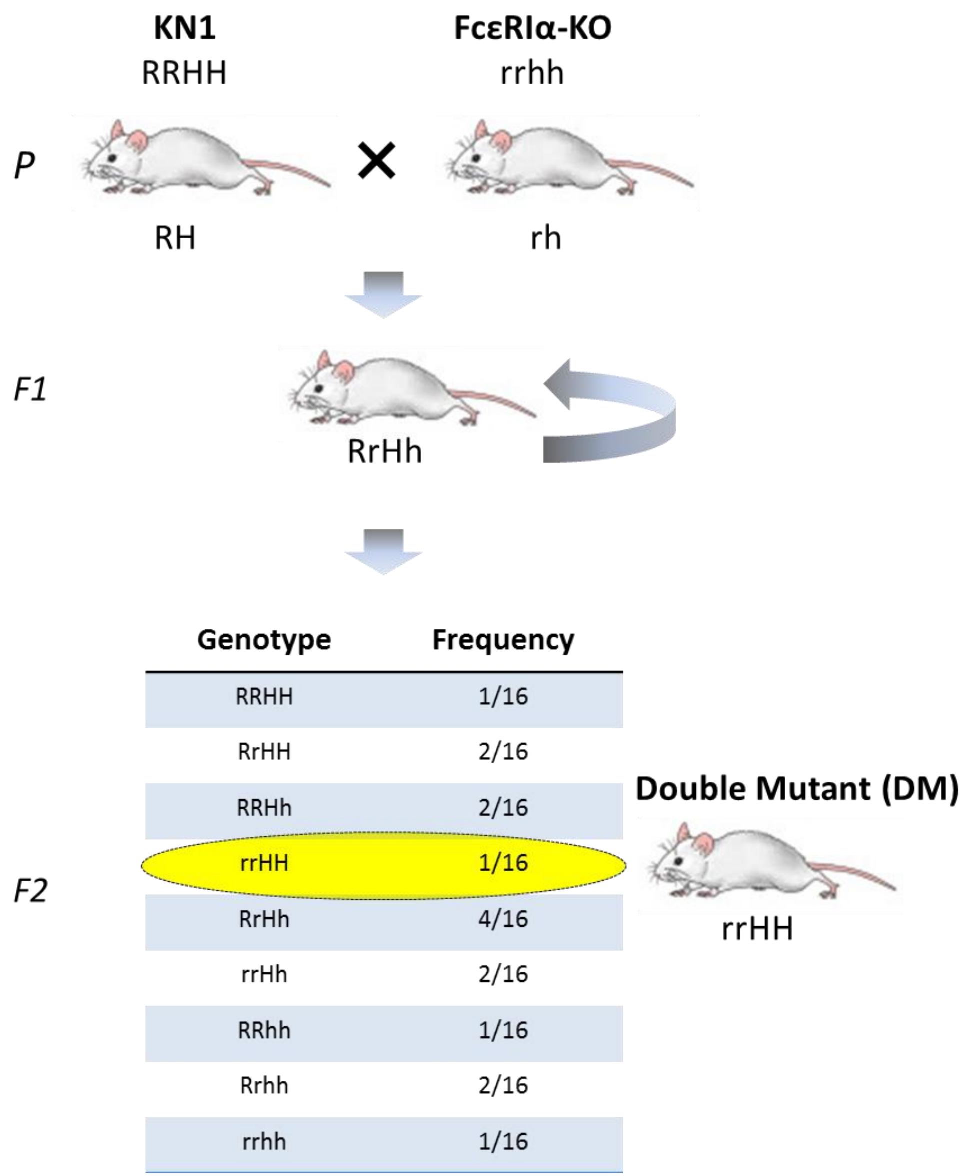
Figure 5. Metastases do not develop in KN1 mice. After 35 days from i.v. injection of $1.2x10^6$ N2C tumor cells, lungs were analysed macroscopically. No tumors were detected in KN1 mice (**A, C**) in contrast to WT mice in which a large number of metastases is observed (represented by white patches) (**B, D**). Haematoxylin&eosin staining of lungs section are also shown; no tumors are detected in KN1 mice even within the lungs (**E**) compared to WT mice that showed metastases also inside the tumor mass (represented by yellow arrows) (**F**).

The double mutant model

Since IgE perform their role through interaction with their high affinity receptor FcεRI expressed on basophils and mast cells, we wanted to investigate whether the interaction IgE-FcεRIwas actually responsible for the anti-tumor protection observed in the previous experiments in KN1 mice. In order to demonstrate the hypothesis that the antitumor effect was drivenby the interaction of IgE with the FcεRI receptor, we eliminated the high-affinity receptor in the KN1 murine model. This experiment was initiated by Dr. Vijay Yenagi who performed the crosses to obtain double-mutant mice; I was then involved in the genotyping the mice.

KN1 mice were crossed with FεRIα-KO mice (Figure 6), which express the murine receptor FcεRI deprived of the α chain responsible of the interaction of the receptor with IgE and, therefore not able to activate the cells on which this receptor is expressed (mast cells and basophils).

For convenience, the genotype of KN1 mice was indicated as "RRHH", since these mice are homozygous for the WT allele of the high affinity receptor (R) and are homozygous for the mutant allele for the production of high levels of IgE in the serum (H) (Fig. 6); FεRIα-KO mice were named “rrhh”, since these mice do not express the alpha chain of theFcεRI receptor (r) and produce standard amount of IgE (h) (Fig. 6). As expected, in the first generation *F1* we obtained 100% of mice with a genotype “RrHh” (Fig. 6); subsequently, we crossed RrHh mice with each other to obtain a second generation *F2*, consisting of the following combination: 1/16 RRHH, 2/16 RrHH, 2/16 RRHh, 4/16 RrHh, 2/16 rrHh, 1/16 RRhh, 2/16 Rrhh, 1/16 rrhh and finally, 1/16 rrHH (the double mutant, DM) as schematically shown in the lower table of figure 6 (Fig.6).



Genotype	Frequency
RRHH	1/16
RrHH	2/16
RRHh	2/16
rrHH	1/16
RrHh	4/16
rrHh	2/16
RRhh	1/16
Rrhh	2/16
rrhh	1/16

Figure 6. Generation of DM (rrHH) mice. Parental KN1 (RRHH) and FcεRIα-KO (rrhh) mice (P) were crossed to generate *F1* mice (RrHh). Crossing RrHh mice we obtained all combinations of F2 generation, including the DM mice as shown in table below.

To select DM mice to be enrolled in subsequent experiments, from each animal of the *F2* generation a tai biopsy was performed to extract DNA and evaluate the genotype by PCR (Fig. 7); animals expressing the “H” allele and the “r” allele and not expressing the “h” allele and the “R” allele, were selected for subsequent crossing between them (Fig. 7),in order to expand the number of this DM group. The new mouse model obtained, has always shown a good vitality, has never shown signs of infertility and we never observed symptoms of any disease.

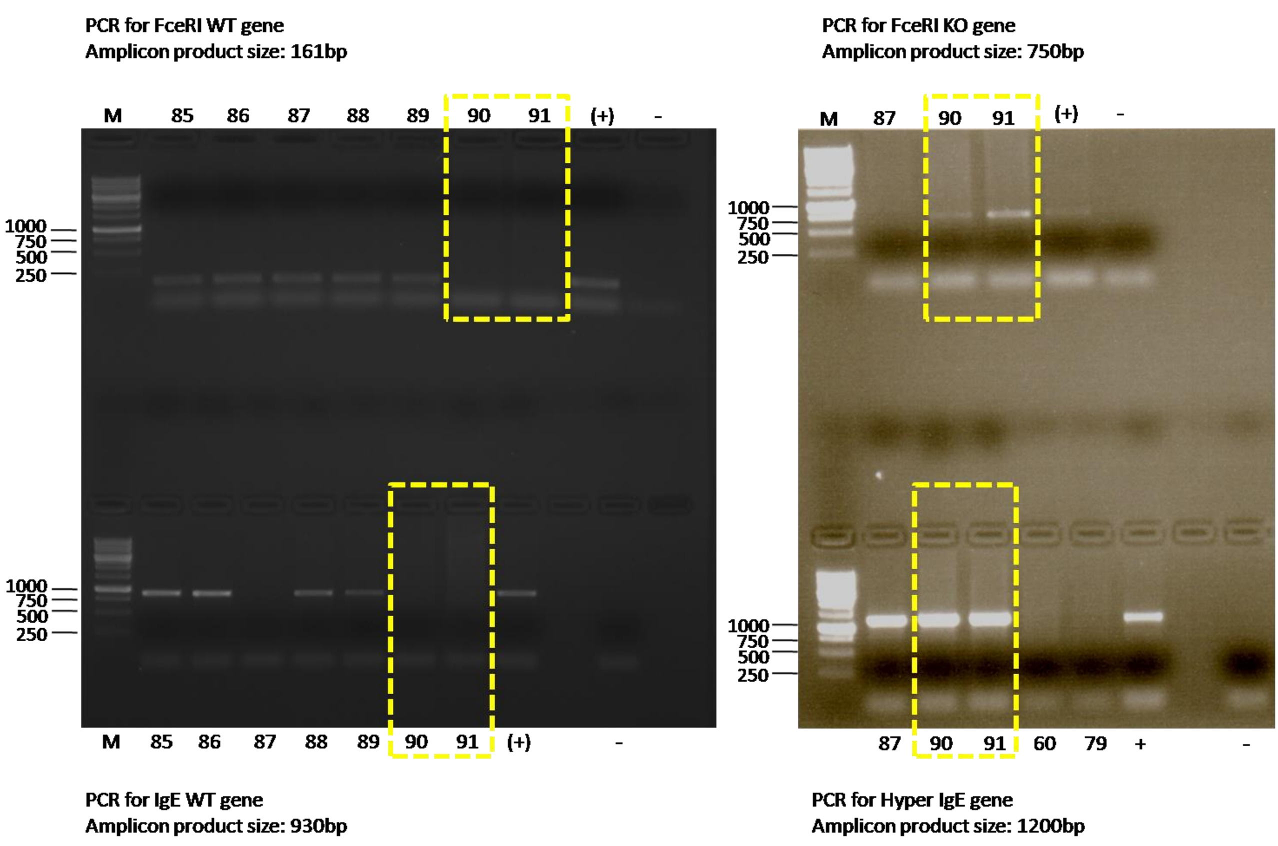


Figure 7. PCR genotyping of all the expected genotypic combinations in the *F2* progeny, including the DM mice. Representative gels of PCR product; DM mice (90 and 91) are characterized by the absence of FcεRI wild type allele (amplicon product size 161bp) and IgE wild type allele (amplicon product size 930 bp) and are characterized by the expression of FcεRIα KO (amplicon product size 750 bp) and expression of allele for the high level of IgE (amplicon product size 1200 bp).

Loss of antitumor protection in DM mice

KN1, FεRIα-KO, DM and WTmice were treated subcutaneously with $6x10^5$ N2C tumor cells. In this experiment the tumor growth and survival of animals were monitored; furthermore, from each animal blood was collected before tumor cells implantation and at day 15, 30 and 45 post tumor cells injection, in order to evaluated the presence of tumor specific IgE in the serum.

As shown in Figure 8, the anti-tumor protection previously observed in KN1 model, was almost completely lost in DM murine model, in fact, after 44 days post tumor cells implantation, the tumor mass volume in the double mutant mice were significantly greater compared to that in KN1 mice (Fig. 8-A). Interestingly, tumors in the double mutant mice had grown significantly less than the tumors in WT mice (Fig. 8-A); this could be due to the fact that even if DM do not express the FcεRIα chain, high level of IgE might be able to engage the low affinity receptor CD23. However this possibility was not further investigated. The trend of survival largely reflects what has been observed in tumor growth, although the difference in survival between the DM mice and WT mice does no reach the level of statistical significance (Fig. 8-B).

The data provide evidence that the interaction of IgE with FcεRI receptor is essential for the anti-tumor mechanism implemented by IgE.

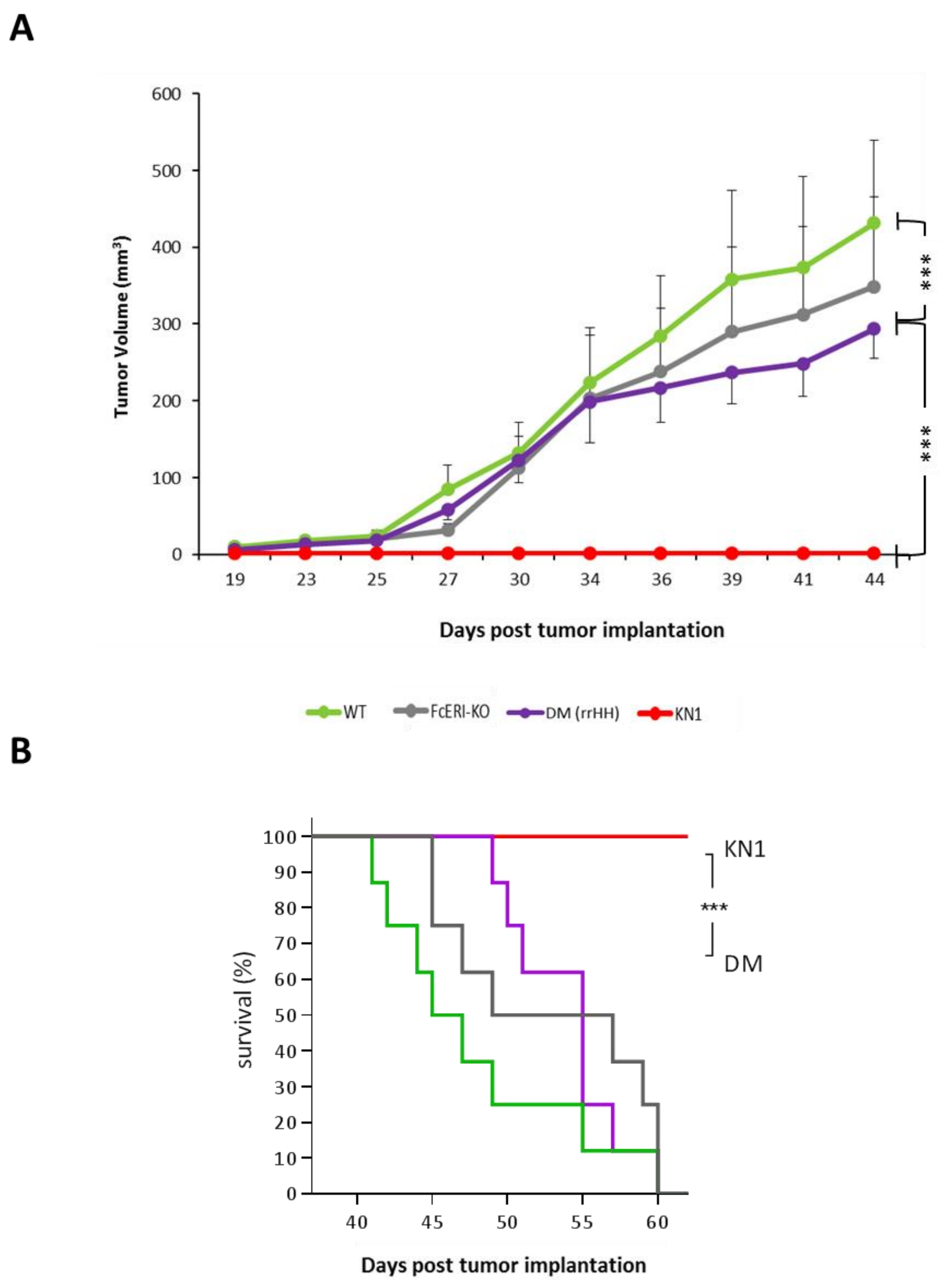


Figure 8. Tumor protection mediated by IgE requires the FcεRI expression. **A**, tumor growth curves of WT (green line), FcεRIαKO (grey line), DM (purple line) and KN1 (red line) mice challenged with N2C cells. **B**, survival of mice in A. The experiment shown indicates 3-4 replicates. *n*=5 mice. Error bars indicate SD. **P*< 0.05; ***P*< 0.01; ****P*< 0.001.

The observation of tumor growth in all the mice of the *F2* generation showed that animals which preserve at least one H allele and one R allele (animals with RRHh, RrHh and RrHH genotype) had an intermediate protection against N2C tumor growth (Fig. 9). Indeed, these three groups showed a significantly lower tumor growth compared to the block which includes the parental FεRIα-KO (and its equivalent rrhh), RRhh (equivalent of WT mice), Rrhh and rrHh groups (Fig. 9).

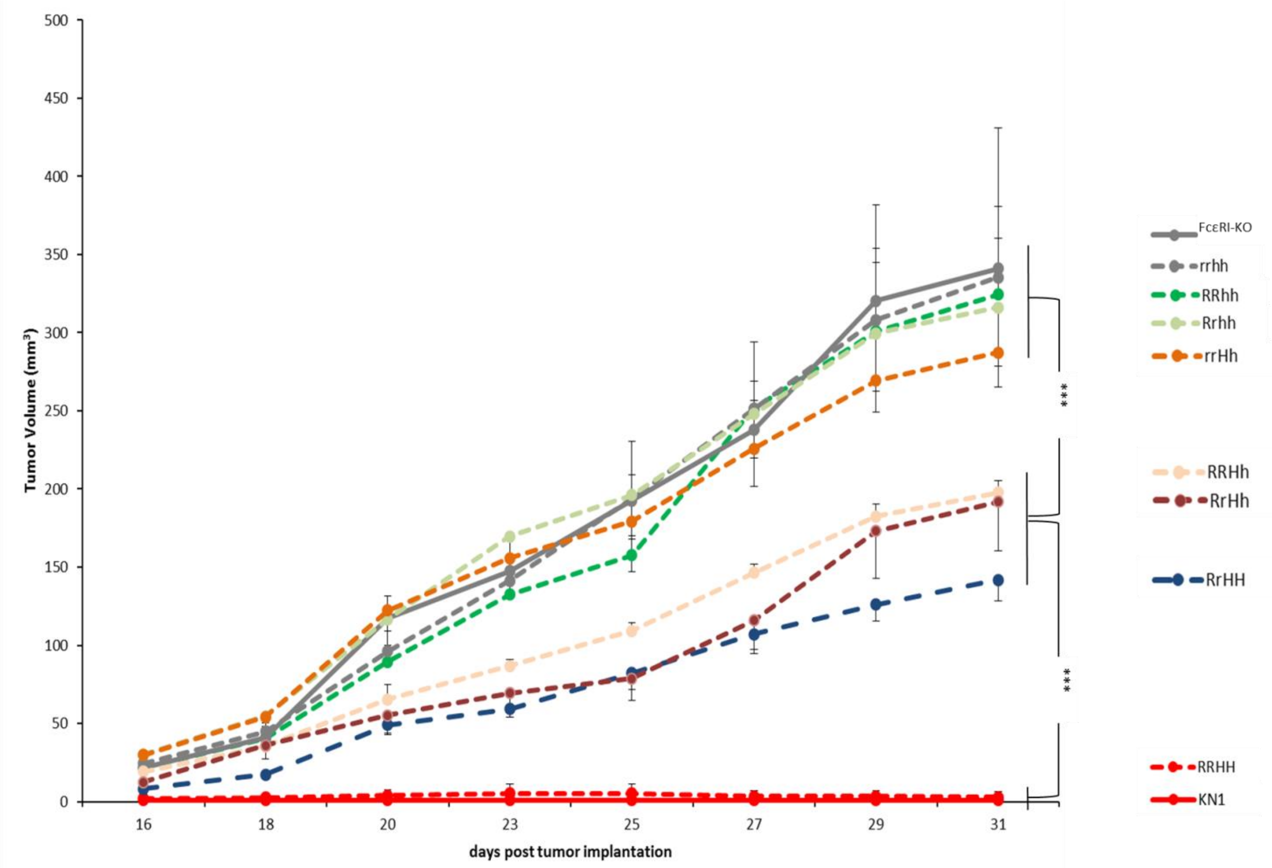


Figure 9. The antitumor protection is proportional to the expression of IgE and its receptor. N2C tumor growth in the parental strains (FcεRIα-KO and KN1) and in all *F2* mice genotypes (except DM), grouped in 3 clusters: all mice with a hh genotype (FcεRIα-KO, its F2-equivalent rrhh, the WT-equivalent RRhh, Rrhh), plus rrHh; mice with at least one R allele and one H allele (RrHH, RRHh and RrHh); mice with a RRHH genotype (KN1 and its F2-equivalent).

Detection of tumor-reactive IgE

The results obtained so far showed that tumor immunosurveillance was very elevated in KN1 (high IgE producer) mice, suggesting a probable production in these mice of tumor-specific IgE in response to challenge with tumor cells.

As previously mentioned, from each KN1, FcεRIα-KO,DM and WT mouse of the experiment showed in figure 8, blood was collected before N2C implantation and after 15, 30 and 45 days from tumor implantation, in order to evaluated, by a β-exosaminidase release functional assay, the presence of tumor-specific IgE in the serum. The rationale of the assay is that tumor-reactive IgE bind to antigens on the surface of N2C tumor cells and then interact with FcεRI receptors expressed on the surface of RBL-2H3 cells (Rat Basophilic Leukemia cells), with consequent release of mediators of allergic reaction, including β-hexosaminidase. For this reason N2C tumor cells have been incubated with sera from WT, FcεRIα-KO, KN1 and DM mice and subsequently incubated with the RBL-2H3 cells. As shown in figure 10, if N2C tumor cells were incubated with IgE/k isotype control antibody there was no significant release of β-hexosaminidase; cross-linked IgE on the contrary gave a release of β-hexosaminidase (represented as 100% = positive control of the assay) (Fig. 10). The sera from WT and FεRIα-KO mice did not produce any significant release of β-hexosaminidase at any time (Fig. 10). On the contrary, at 45 days post N2C challenge, sera from KN1 and DM showed a release of β-hexosaminidase significantly higher than the sera of the same animals, collected before tumor cells implantation (Fig. 10). A second inoculation of tumor cells showed a persistence of tumor specific IgE in the sera of KN1 mice.

Finally, if IgE in sera of KN1 and DM groups were removed (by depletion), the release of mediator of the allergic response was significantly abolished (Fig. 10) demonstrating the specificity of the response.

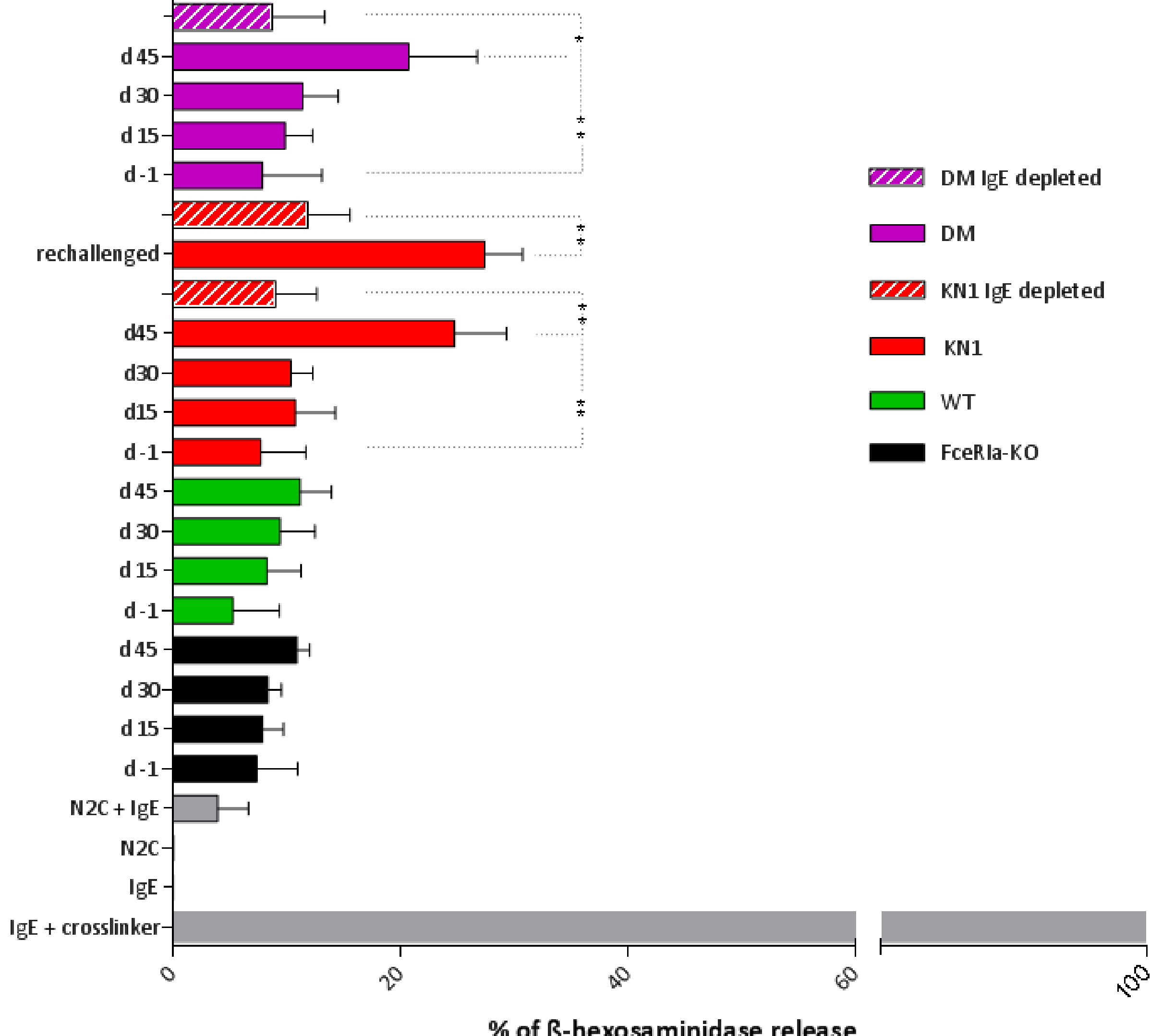


Figure 10. Tumor-specific IgE induce the β-hexosaminidase release by RBL-2H3 cells. Sera from FcεRIαKO, WT, KN1 and DM mice were collected before and after implantation of N2C tumor cells. Sera of KN1 and DM mice at 45 days after challenge, and, for KN1, 30 days after a second tumor challenge induce a significant release by RBL-2H3 cells. Depletion of IgE in sera of KN1 and DM groups significantly abolished the release of β-hexosaminidase. Cross-linked IgE gave a release of β -hexosaminidase that was represented as 100% (positive control). This figure is a representative experiment out of 3 replicas. *n* = 3 mice. Error bars indicate SD. *P< 0.05; **P< 0.01.

Isolation of tumor-specific IgE

In order to isolate tumor-specific IgE, KN1 mice were challenged i.p. with N2C tumour cells. Spleen and bone marrow cells were collected and fused with NS1 murine myeloma cells to produce hybridomas. The cell suspension were incubated for 10-15 days and subsequently, the supernatants of wells showing hybridoma growth were analyzed for the presence of IgE by ELISA test using an anti-IgE antibody. To evaluate the sensibility of the ELISA test we performed a decreasing concentration curve of a commercial standard IgE showing that the assay was sensitive up to a concentration of 31.3 ng/ml of IgE (data not shown). ELISA test proved that out of 87 supernatants, selected from the wells that showed cell growth, we could detect only three IgE-producing hybridomas. These clones were signed as DG5, IG5 and TG3 (Fig.11).

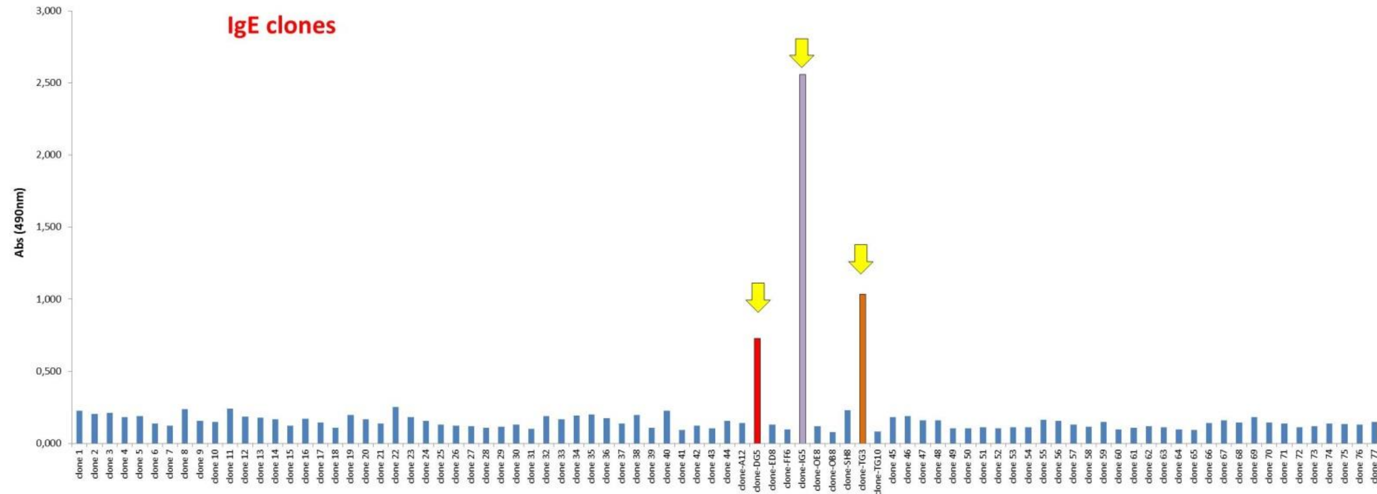


Figure 11. Identification of tumor specific IgE. Supernatants of hybridomas were analyzed for the presence of IgE by ELISA test using an anti-IgE antibody. Out of the 87 tested hybridomas supernatants, only three IgE-producing ones were detected .

Tumor-specificity assessment of selected clones

Subsequently, we evaluated the tumor-specificity of the selected IgE-producing hybridomaclones. Therefore, N2C tumor cells were incubated with supernatants derived by DG5, IG5 and TG3 clones and, subsequently, with RBL-2H3 cells (Fig. 12). In order to be sure of the clonality of the hybridomas, before this specificity assay, we performed two subcloning of the selected clones. Unfortunately, none of the selected clones showed specificity towards N2C tumor cells, since the release of β-hexosaminidase was never significant (Fig. 12).

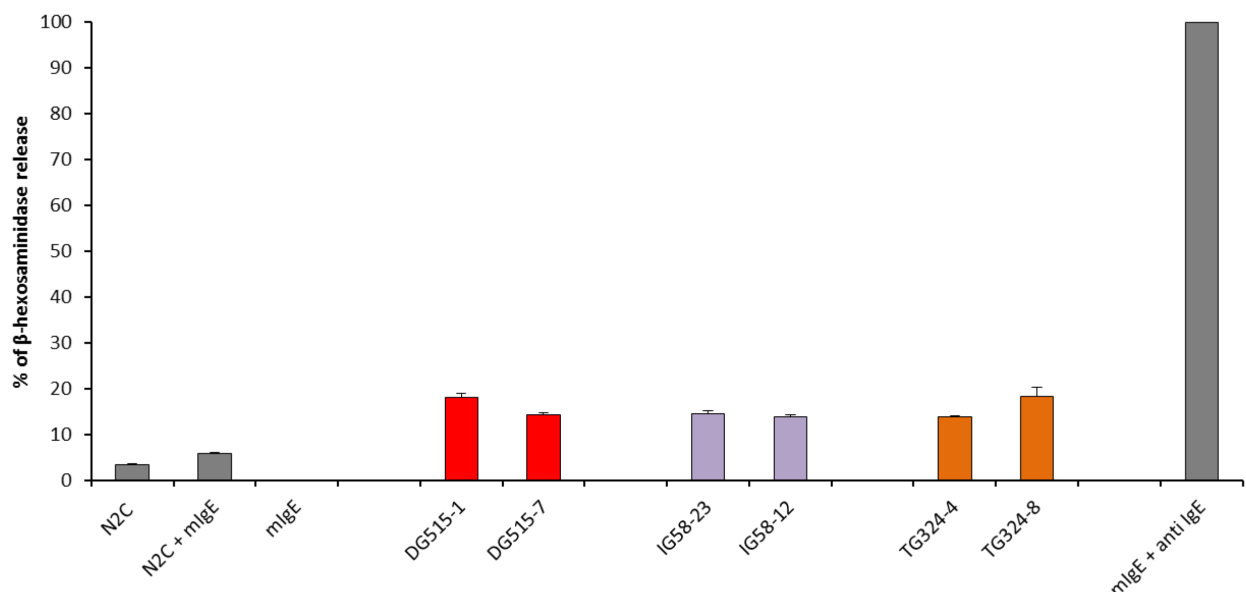


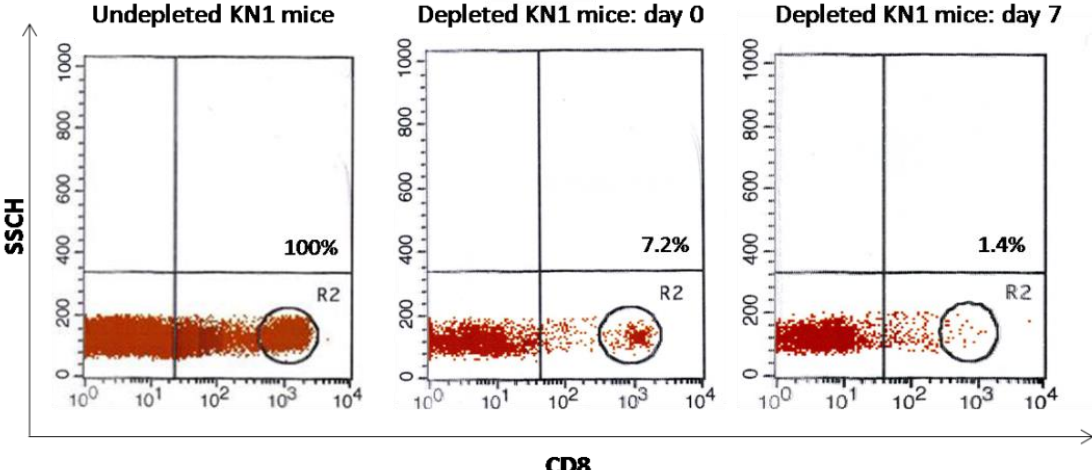
Figure 12. Supernatants of IgE-producing hybridomas selected are no able to induce the release of β-hexosaminidase by RBL-2H3 cells. None of the selected clones (DG515-1, DG515-7, IG58-23, IG58-12, TG324-4, TG324-8) induce any significantly release of β-hexosaminidase compare to the negative control (N2C tumor cells incubated with standard IgE).

CD8$^+$ T cells involvement in IgE-dependent anti-tumor protection

After showing the role of IgE in tumor immunosurveillance we started looking deeper into the mechanism leading to tumor protection. According to our previous studies (Reali E et al. 2001) on IgE adjuvanticity in tumor vaccination, the IgE-driven anti-tumor protection is dependent on eosinophils, CD4+ and CD8+ T cells. In order to test the involvement of CD8+ T cells in cancer immunosurveillance in presence of high levels of IgE, we depleted CD8$^+$T cells in KN1mice, through 3 consecutive injections (at day 6, 3 and 1 before tumor challenge) of anti-CD8 antibody before implantation of N2C tumor cells. Subsequently, the depletion of CD8 + T cells was monitored by cytofluorimetry on peripheral blood.

As shown in Figure 11, on day 0 approximately 90% of CD8$^+$ T cells had been depleted and after 7 days over 95% of these lymphocytes were eliminated; as control the peripheral blood of KN1 mice not depleted of CD8$^+$ was used (Fig. 13-A). Interestingly, depletion of CD8$^+$ T lymphocytes in KN1 mice dramatically abolished the tumor protection observed in not depleted KN1mice (Fig. 13-B).This finding demonstrated that the tumor immunosurveillance, observed until now in our experiments, depends on CD8$^+$ lymphocytes.

A



B

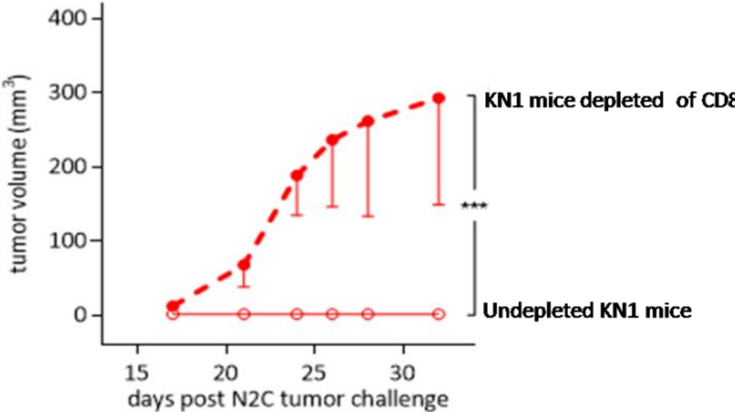


Figure 13. Involvement of CD8+ T cells in tumor protection driven by IgE. A, FACS analysis confirm the depletion of CD8+ T cells in blood collected from KN1 mice. Compare to undepleted mice, the depletion in KN1 depleted mice was >90% at day 0, and > 95% at day 7 post treatment with the anti-mouse CD8 antibody. **B,** KN1 mice depleted of CD8 show a significantly tumor growth compared with undepleted KN1 mice demonstrating the involvement of CD8+ T cells in antitumor protection.

In vitro test of cancer cells infected with MVA-HcRED

Since we were unable to isolate an hybridoma producing tumor specific-IgE to be employed in therapeutic protocols in mice, we decided to employ the MVA-tmIgE vaccine in therapeutic protocols for the intratumoral treatment of solid tumor mass.

This MVA virus (Modified Vaccinia Virus Ankara) expressing truncated, but functional human membrane IgE was previously produced in our laboratory, and has already been used in vaccination protocols (Nigro et al 2012).

Before using the MVA-tmIgE, a control virus MVA-HcRED which express a red fluorescent protein was tested *in vitro* to assess the infection of TS/A tumor cells.For this purpose, $2x10^5$ TS/A cells were infected at different MOI with the virus and, the next day, the infection success was testedusing fluorescence microscopy for the identification of cells emitting red fluorescence. As positive control of infection, CEF cells infected at MOI 1 with MVA-HcRED were used. The infection of the TS/A cells was successful, in fact, 24 hours after the infection, TS/A tumor cells expressed the red fluorescence given by the expression of the HcRED fluorescent protein (Fig. 14). In addition, infection was proportional to the MOI of virus added to cell culture (Fig. 14).

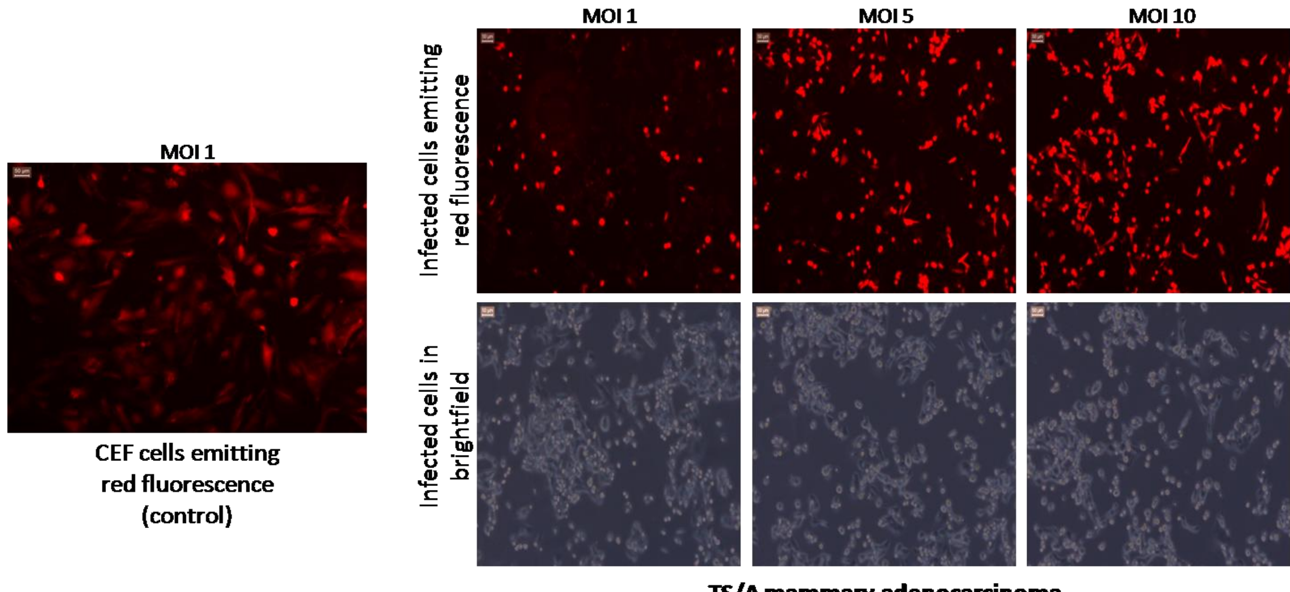
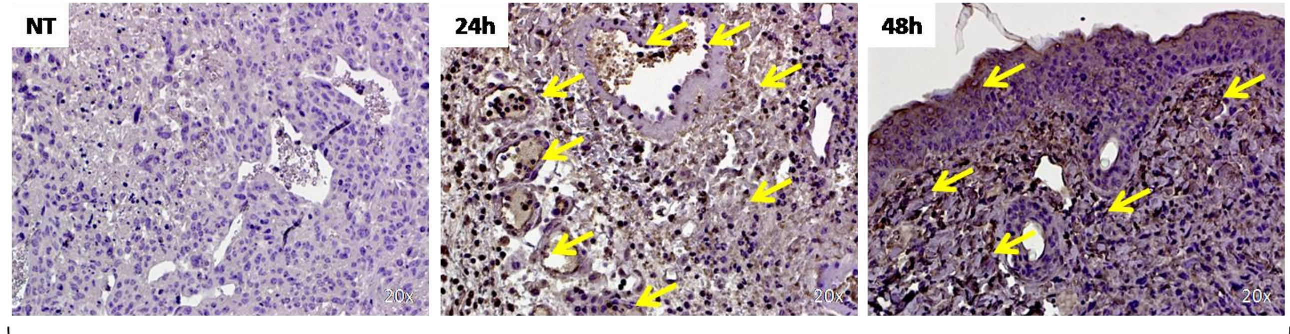


Figure 14 .MVA-HcRED is able to infect TS/A tumor cells. $2x10^5$ TS/A cells were infected at 1-5 and 10 MOI of MVA-HcRED. After 24 hours from infection, TS/A tumor cells were able to express the red fluorescence given by the expression of the HcRED fluorescent protein. As shown in figure, infection was proportional to the MOI of virus added to cell culture (right panel). As positive control of infection, CEF cells infected at MOI 1 with MVA-HcRED, were used (left panel).

Intratumoral treatment: preliminary data with MVA-HcRED

In order to understand whether the MVA-HcRED virus was able to infect cells within a tumor mass, we performed an *in vivo* experiment of intratumoral infection. To achieve this aim, mice received a dose of $2x10^5$of TS/A tumor cells, injected subcutaneously on the abdominal wall; when the tumor mass was palpable (100-200mm^3 of volume), it was treated with10^7 pfu of MVA-HcRED by intratumoral injection and after 24 and 48 hours from treatment, the infection of cells into the tumor mass was tested through immunohistochemistry assay.

IHC assay showed that MVA-HcRED was able to infected the cells into the tumor mass (Fig. 15); the signal of infection (represented by brown staining) was evident at 24 and 48 hours after intratumoral treatment (Fig. 15); as negative control of the experiment we used a tumor mass treated with PBS and, as expected, in this case we had not obtained any signal of infection as shown in figure 15.



anti- Vaccinia Virus

Figure 15. MVA-HcRED is able to infect cells within the tumor mass. IHC assay, for the presence of vaccinia virus show that MVA-HcRED infect the cells within the tumor mass after 24 and 48 hours from intratumoral injection of MVA-HcRED. The signal of infection are represented by brown staining (indicated with yellow arrows); as negative control of the experiment was used a tumor mass treated with PBS (NT).

In vitro test of cancer cells infected with MVA-tmIgE

Before assessing the ability of the rMVA-tmIgE to infect cells within a TS/A tumor mass, we evaluated the infection of TS/A tumor cells *in vitro*. To this end $2x10^5$ TS/A cells, infected with rMVA-tmIgE at 1, 5 and 10 MOI, was assayed for the expression of human mIgE on cell surface, through cytofluorimetry using an anti-human IgE antibody as tracer.

After 24 hours from infection, cytofluorimetry demonstrated that rMVA-tmIgE virus was able to infect tumor cells and express the human membrane IgE on the cell surface (Fig. 16). Furthermore, the infection was proportional to virus MOI used to infect TS/A tumor cells *in vitro* (Fig. 16).

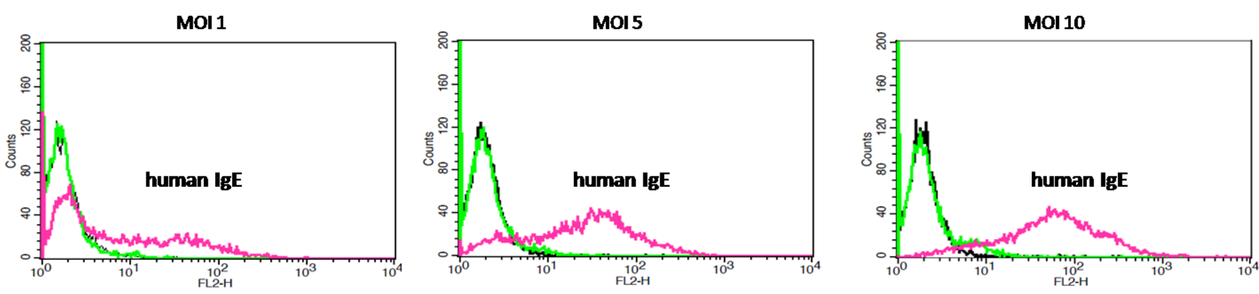


Figure 16. FACS analysis for the expression of human IgE in TS/A tumor cells infected with rMVA-tmIgE virus at 1, 5 and 10 MOI. 24 hours after infection, tumor cells express human membrane IgE on their surface (pink line). The expression is proportional to MOI of virus used as shown in figure. Negative controls are both uninfected TS/A (black line) and infected TS/A incubated with only secondary antibody (green line).

Intratumoral treatment with rMVA-tmIgE

Next we tested the rMVA-tmIgE ability to infect cells within the tumor mass. This experiment was performed to evaluate the capacity of rMVA-tmIgE to express *in vivo* the human membrane IgE on the surface of tumor cells after intratumoral injection of the virus. Mice received a dose of $2x10^5$ TS/A tumor cells, injected subcutaneously on the abdomen and then the tumor mass was treated with 10^7pfu of rMVA-tmIgE by intratumoral injection. Subsequently, after 24 and 48 hours from treatment with the virus, the tumor mass was harvested and the infection of cells into the mass with the expression of human IgE on the cells surface was tested through immunohistochemistry assay using an anti-vaccinia virus and an anti-human IgE antibodies, respectively.

IHC assay showed that rMVA-tmIgE was able to infected the cells into the tumor mass already after one day from infection (Fig. 17-A); the signal of infection (represented by brown staining) was evident at 24 and 48 hours after intratumoral treatment (Fig. 17-A). Considering the ability of rMVA-tmIgE to express the human IgE *in vivo*, the signal of human IgE expression is evident at 24 and 48 hours after intratumoral treatment(Fig. 17-B), even if the signal of expression is much lower compared to the signal of infection (Fig. 17-A,B). As negative control of the experiment, a tumor mass treated with PBS was used and, as expected, also in this case we did not obtain any signal of infection or human IgE expression.

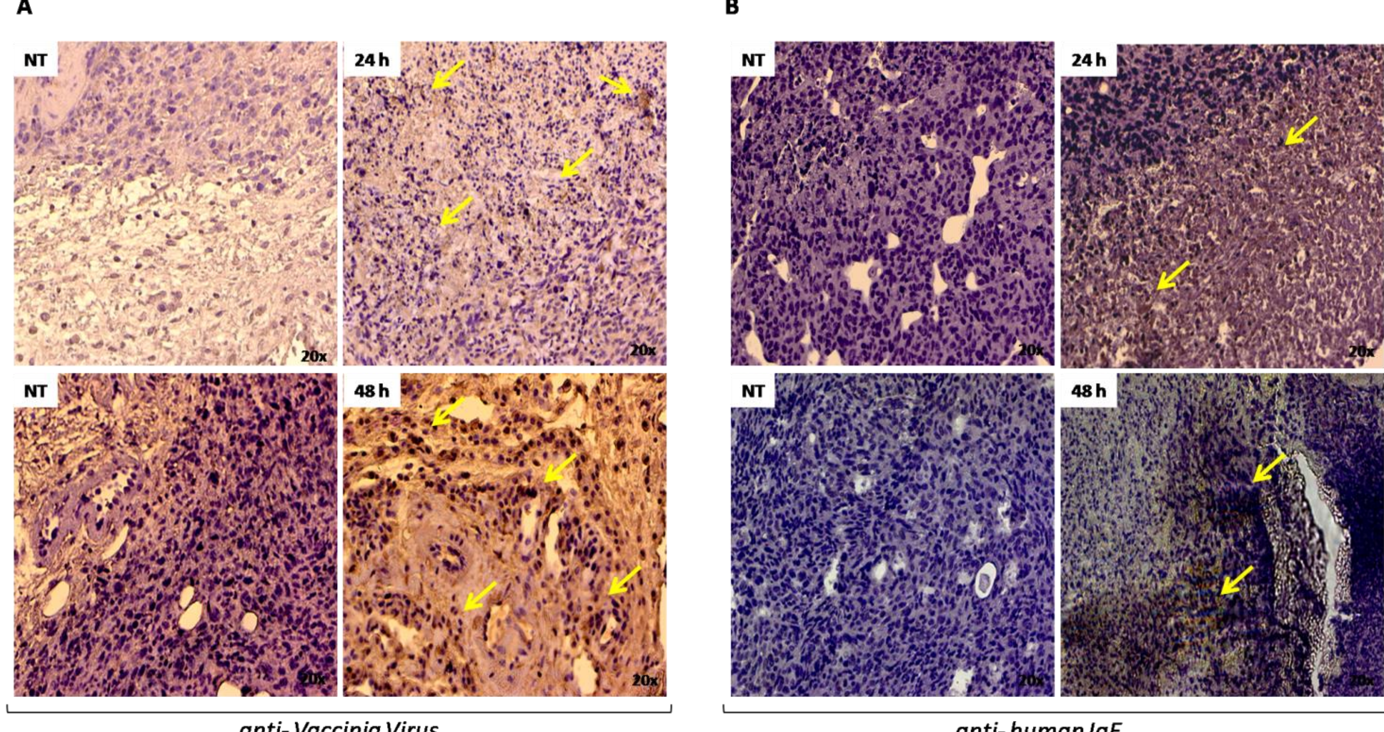


Figure 17. In N2C tumor mass, cells infected with MVA-tmIgE express the human IgE on their surface.A, IHC assay, for the presence of vaccinia virus show that MVA-tmIgE is able to infect, *in vivo,* cells within the tumor mass after 24 and 48 hours from intratumoral treatment. **B**, MVA-tmIgE express the human IgE on infected cells surface. The expression was detectable already after 24 and 48 hours post intratumoral treatment. The signal of infection or human IgE expression is represented by brown staining (indicated with yellow arrows); negative control is represented by tumor mass treated with PBS (NT).

DISCUSSION

The potential of IgE to induce an anti-tumor protection is the basis of the innovative research field called *Allergo-Oncology* (Jensen-Jarolim E et al. 2008; Singer J and Jensen-Jarolim J 2014).

Various studies have demonstrated the capacity of IgE to induce of tumor cells and to prevent tumor growth (Karagiannis SN et al. 2008; Karagiannis P. et al. 2009; Daniels TR et al. 2012; Nigro EA et al. 2013; Josephs DH et al. 2014). All studies related to this research area were inspired by epidemiological data that suggest allergies as a factor that can reduce the incidence of certain types of cancer, such as pancreatic tumor and glioma (Turner MC et al. 2006; Leoh LS et al. 2015).According to these epidemiological data, the allergic condition would favor the prevention of tumor development; however, this is highly dependent on the type of tumor considered, and on the type of allergic disorder.

These studies clearly indicate that are-direction of IgE on tumor cells surface, is able to determine cellular cytotoxicity. The re-direction of IgEwas made through the development of monoclonal IgE specific to tumor antigens such as, to the mouse mammary tumor virus (MMTV) (Nagy E et al. 1991) or to the antigen specific to an antigenic determinant expressed on the surface of human adenocarcinoma of colon-rectal cells as COLO 205 (Kershaw MH et al. 1998).

In addition, the construction of a chimeric human-mouse monoclonal IgE called MOv18, specific for the FBP(Folate-binding protein) expressed on ovarian carcinoma cells (Gould HJ et al. 1999), allowed to demonstrate the involvement of human monocytes as anti-tumor effectors cells in a tumor xenograft model performed in scid mice (Karagiannis SN et al. 2003).

In an initial work of our group the ability of mouse IgE, loaded on the surface of tumor cells, to influence the tumor growth and to give protection in terms of anti-tumor vaccination it has been demonstrated in C57BL/6 mice using two type of tumor cells: a cell line of adenocarcinoma and a T lymphoma cell line (Reali E et al. 2001). In this study, the loading of IgE was obtained through the 3-step strategy, based on the biotin-avidin bridge, performed both *in vitro* prior inoculation in mice or*in vivo.* The use of the 3-step strategy eliminated the need to build specific IgE against tumor antigens. The vaccination protocol consisted in inoculation of two doses of irradiated tumor cells, loaded with IgE or IgG, followed by administration of live tumor cells at 15 days after the last immunization. Vaccinated mice showed a strong protection against tumor growth in the group vaccinated by IgE-loaded tumor cells, indicating the formation of an adjuvant effect driven byIgE.

Since the inoculation of live tumor cells occurred in the absence of IgE, the protective effect driven by IgE was to be attributed to the stimulation of the immune system by prior vaccination with tumor cells loaded with IgE. Moreover, the presence of eosinophils, CD4+ and CD8+ T cells had been shown to be crucial for the establishment of anti-tumor immunity; in fact, their depletion brought to the abolition of anti-tumor protection even in mice immunized with IgE-loaded cells (Reali E et al. 2001).

Subsequently it was demonstrated, in animal models, the involvement of IgE in anti-tumor immune response through a process that involves the interaction of IgEwith the high affinity receptor FcεRI. In this study (Nigro EA et al. 2009), it was observed that the IgE driven anti-tumor protection observed in wild type mice was completely lost in mice knock-out for αFcεRI receptor. This result indicated the crucial role played by the high affinity FcεRI receptor in the anti-tumor effect driven by IgE.

The activation of FcεRI receptor, expressed on murine mast cells and basophils, leads to a potent degranulation of these cells and results in the release of chemotactic mediators such as histamine, proteoglycans, β-hexosaminidase, thromboxane and leukotrienes; mediator release induces the recruitment of effector cells, responsible for the development of a powerful inflammatory process (Mukai K et al. 2005).In terms of anti-tumor applications, the development of local inflammation would result in tumor cell death and processing of tumor antigens, thus causing the onset of a strong immune response against tumor. Furthermore, the use of a transgenic mouse model, knock-out for the α chain of the murine FcεRI receptor and knock-in for the human α chainFcεRI$\alpha^{-/-}$ hFcεRIα^{+} receptor (Dombrowicz D et al. 1996), allowed to continue the studies using human IgE, a fundamental step for a possible clinical application of IgE-driven anti-tumor vaccination. Human IgE showed an adjuvant effect in anti-tumor vaccination in “humanized mice”, but not in wt mice, highlighting the species-specificity of IgE-receptor interaction (Nigro EA et al. 2009).

Based on these results, in this thesis project we wanted to investigate whether endogenous IgE also had an anti-tumor adjuvant effect, given that previous studies were related onexogenous IgE only.

For this purpose, we employed IgE-KO mice that are characterized by the absence of the transmembrane and cytoplasmic domains of IgE and in which serum IgE levels are reduced of

about 94-98% due to a smaller number of IgE-secreting plasma cells (Achatz G et al. 1997).The s.c. inoculation of TS/A tumor cells in IgE-KO and WT mice showed a very similar growth.

In parallel, we decided to immunize some IgE-KO and WT mice with irradiated TS/A tumor cells prior to challenge mice with live tumor cells. In this case the tumor growth in WT mice was significantly decreased and the survival was prolonged respect to not immunized WT mice. Differently, tumor growth and survival in immunized IgE-KO mice was absolutely not changed compared to non-immunized mice. This result indicates that the protection observed in immunized WT mice depends on the anti-tumor IgE response induced by immunization.

After documenting the effect of the loss of IgE expression, we investigated how the system would act, instead, in the case of an increase of IgE concentration. We used a transgenic mouse model called KN1; this transgenic strain expresses a chimeric ε-γ1 BCR, consisting of the extracellular domain of the ε gene and the transmembrane and cytoplasmic domains of the γ1 gene. The resulting antibody is a true IgE but is characterized by a γ1 transmembrane domain anchored on the B cell which, unlike the ε transmembrane domain, causes an alteration in homing and an increase in survival of plasma cells. The KN1 mice model was very suitable for this part of the project since, in these mice, the number of IgE-secreting B cells increases up to 10 times in the bone marrow and the levels of IgE in the serum increase by 4-6 times compared to WT mice, after interaction with the antigen (Achatz-Straussberger G et al. 2008).

KN1 mice, immunized with irradiated TSA cells and then challenged with live tumor cells, displayed no tumor growth and presented 100% of survival; moreover, if tumor cells were inoculated without prior immunization, tumor growth in KN1 mice was still much lower than the growth observed in WT andIgE-KO mice. To confirm this data, in addition to TSA tumor cells, we introduced another type of tumor cells in our experiments: the N2C tumor cells, a primary mammary carcinoma cell line derived from BALB-Neu T mice (Melani C. et al. 2003), which are less aggressive than TSA tumor cells. Using N2C tumor cells, a complete protection against tumor growth in KN1mice was observed even without prior immunization, and the survival rate was 100% (at difference from WT and IgE-KO mice). Furthermore, on some KN1 mice we performed a second challenge with tumor cells, but we never observed any tumor growth in any KN1 mice (data not shown).

IgE exerts its role through the interaction with its two receptors, the high affinity FcεRI receptor (Kinet JP 1999)which in mice is expressed on mast cells and basophils, and the low affinity CD23

receptor expressed on B cells and on antigen presenting cells (Kijimoto-Ochiai S 2002). A previous study had already shown that the effect of IgE in anti-tumor vaccination depends on the high affinity receptor and not on the low affinity receptor (Nigro EA et al. 2009).

To demonstrate that the protection observed in KN1 mice was due to the interaction of IgE with FcεRI, we planned to remove the high affinity receptor in these mice. To this purpose, we crossed KN1 mice with FcεRIα-KO mice, in order to obtain a double mutant mice (DM) characterized by high levels of IgE, but lacking the high-affinity receptor.

The challenge with N2C tumor cells in DM mice showed that the antitumor protection previously observed in KN1mice, was largely lost, clearly demonstrating that the IgE-FcεRI interaction is fundamental for IgE-driven anti-tumor immune surveillance. It should be emphasized that the tumor growth in DM mice was anyway significantly lower than in WT mice, and this may be due to the fact that IgE present at high levels in DM, could have activated alternative protective mechanisms. The activation of alternative mechanisms may also explain the fact that tumor growth in IgE-KO and FceRI-KO mice is not the same as it would be expected if the IgE-FcεRI axis were the only involved in the IgE-driven antitumor effect driven by IgE. Therefore, a concomitant minor role played by CD23 might not be excluded. However, no experimental results are available, since they would require the lenghty and cumbersome construction of double mutants.

The fact that the immune-surveillance in IgE-KO mice is strongly decreased and in KN1 mice is greatly increased suggests that in these mice the activation of an antitumor response driven by IgEis a consequence of the tumor cells challenge.

Therefore we tried to detect tumor-specific IgE in sera of both KN1 mice and DM mice challenged with N2C tumor cells. Since conventional immunoassays turned out to be not sensitive enough for this purpose, we employed a very sensitive functional assay previously developed in our laboratory (Vangelista L et al. 2005). Indeed, sera derived from KN1 and DM mice were able to induce a significantly release, *in vitro*, of mediators such as β-hexosaminidase, after incubation with tumor cells, unlike sera derived from WT and FcεRIα-KOmice that did not induce any release of mediators. Furthermore, the depletion of IgE from KN1 and DM sera, *in vitro*, completely abolished the release, validating the contribution of IgE.

This result encouraged us to try to isolate tumor-specific IgE through hybridoma technology, starting from spleen or bone marrow of KN1 mice *in vivo* challenged with the tumor. IgE hybridomas are extremely rare and actually only one of them is commercially available (Eshhar Z

et al. 1980). It was then surprising to obtain 3 IgE hybridomas out of a total of 87 hybridomas. Unfortunately, none of them showed any specificity for the tumor cells. Considering that the frequency of antigen specific hybridomas recovered in any experiment is rather low (however less than 5 %), our results just stress that very large screens, unfeasible in our laboratory limitations would be necessary.

A fundamental issue for this study was to understand how IgE operate to give the observed antitumor effect. In previous studies, it was demonstrated that the adjuvant effect of exogenous IgE in tumor treatment depended on CD8 + T cells (Reali E et al. 2001; Platzer B et al. 2015). Therefore, we have depleted the CD8+ T cells in KN1mice prior to the inoculation of N2C tumor cells; this allowed the growth of the tumor in KN1 mice, demonstrating that the anti-tumor immune surveillance driven by IgE is largely mediated by an immune response dependent of CD8+ T cells.

The mechanism that involves IgE and CD8+ T cells in anti-tumor protection remains to be discovered. In a recent study it was shown that IgE-driven antigen cross-presentation by mouse dendritic cells, modified to express human FcεRI, activated the production of anti-tumor cytotoxic CD8+ T lymphocytes (Platzer B et al. 2015).

Since the high affinity FcεRI receptor is not present on murine dendritic cells, other cells expressing FcεRI must be involved in such mechanisms. Murine mast cells could induce the activation of T cells by releasing exosomal materials, including IgE-antigen complexes (Raposo G et al. 1997; Skokos D et al. 2003).

Many studies (Leoh LS et al. 2015) have tried to prove that the anti tumor immunosurveillance mediated by IgE could be even more effective in humans, since human dendritic cells express the high affinity receptor and may directly participate in the response. The anti-tumor response driven by IgE could be the result of an evolutionary compromise, developed to transmit the anti-parasitic and anti-tumor surveillance activities.

Slight deviations from the "optimal IgE response" may explain the high incidence of atopy, which, in some cases results in a reduced risk of developing cancer (Penichet ML and Jensen-Jarolim E 2010; Josephs DH et al. 2013; Josephs DH et al. 2014).

In the second part of this study, we tried to develop another tool for the treatment of solid tumors. This tool was represented by rMVA-tmIgE (Modified Vaccinia Virus Ankara expressing a truncated form, but functional, of human membrane IgE) previously developed at our laboratories (Nigro EA et al. 2012).

The most important limitation for a possible clinical application of this anti-tumor treatment strategy is the danger of the soluble IgE administration to patients. Even a small fraction ofIgE aggregated in circulation has, in fact, the potential to determine a potent and in some cases fatal anaphylactic reaction. An unexpected recognition of antigens by soluble circulating IgE or IgE already bound to FcεRI receptor can, in fact, lead to the formation of immune complexes capable to activate the receptors present on the surface of mast cells and basophils, resulting in a powerful cell degranulation which leads to systemic anaphylaxis.

To avoid the potential problems of soluble IgE, the use of IgE in their membrane isoform allows to maintain the reactivity at a local level, avoiding any possible systemic complication.

The possibility of employing the membrane IgE (mIgE) relies on previous studies performed in our laboratory on the interaction between human mIgE and FcεRI receptor (Vangelista L et al. 2005).

The study was inspired by structural evidence about the architecture of binding sites of IgE and FcεRI (Vangelista L et al. 2003), by the ability of mIgE to bind the soluble forms of receptor (Yanagihara Y et al. 1994; Vangelista L et al. 2002) and by structural assembly knowledge of mIgE (Bestagno M et al. 2001). It was demonstrated that mIgE are able to bind and activate the FcεRI receptor, independently from the presence of antigen; in the case of human IgE (at difference with murine IgE) this is true also for a truncated portion of mIgE, comprising only Cε3 and Cε4domains (Vangelista et al. 2005).

The employment of rMVA-tmIgE, expressing the Cε3Cε4 portion of a chimeric human membrane IgE on the surface of tumor cells, represents, to our knowledge, the best solution for both efficacy and safety. The rMVA-tmIgE was generated using an innovative engineering approach based on an extremely fast selection, obtained by fluorescence-activated cell sorting approach (red to green gene swapping) (Di Lullo G et al. 2009; Di Lullo G et al. 2010). That rMVA-tmIgE allows to obtain tumor cells infected with MVA and expressing the Cε3Cε4 fragment on cell surface. The impossibility of MVA to replicate in infected cells and then infect host cells surrounding, ensures that the expression of tmIgE-Cε3Cε4 remains confined to the tumor site.

For this reasons, a novel protocol based on rMVA-tmIgE for anti-tumor vaccination was developed (Nigro EA et al. 2012). rMVA-tmIgE was used to infect TS/A-LACK tumor cells *in vitro*. Then infected TS/A-LACK tumor cells were used to vaccinate human FcεRIα mice (FcεRI$\alpha^{-/-}$hFcεRIα^{+}). After fifteen days, vaccinated mice were challenged with live TS/A-LACK cells. This study showed that mice immunized with rMVA-tmIgE-infected TS/A-LACK cells showed a significant attenuation of tumor growth compared to mice immunized with control vaccine not expressing tmIgE (Nigro EA et al. 2012).

On the basis of these data, we conceived that the same IgE-driven effect obtained treating the tumor cells *in vitro* could be obtainable treating the tumors *in vivo* with the same recombinant virus. We therefore planned to perform in situ infection with rMVA-tmIgE which would make the tumor cells express the tmIgE on the surface, transforming the tumor cells in *in vivo* vaccines.

Several studies have demonstrated, in murine models, the efficacy of intratumoral treatment of solid tumors with Vaccinia viral vectors and MVA with a consequent reduction of the tumor mass volume and an increase of survival (Luo D and Saltzman WM. 2000; Paielli DL et al. 2000; Nemeckova S et al. 2007). Intratumoral treatment with viral vectors allows the infection of only residents cells within tumor thus ensuring less dispersion of viral vectors resulting in lower systemic toxicity and in an increase of the effect on tumor site (Paielli DL et al. 2000).

For this reason, in the last part of my project, we have begun to evaluatethe capacity of rMVA-tmIgE to express, *in vivo,* the human membrane IgE on tumor cells surface after intratumoral injection of vaccine in WT mice. The expression of IgE on the membrane of resident cells within the tumor mass is crucial, as, it is the key to trigger an allergic reaction against the tumor.

The tumor mass was treated with 10^{7}plaque forming unit (pfu) of rMVA-tmIgE by an intratumoral injection. After 24 and 48 hours from the treatment, the tumor mass was extracted and both the infection of cells into the tumor mass and the expression of human IgE on the cells surface were tested through immunohistochemistry (IHC). IHC assay showed that rMVA-tmIgE was able to infected the cells into the tumor mass already one day after the intratumoral treatment. Regarding the ability of rMVA-tmIgE to express the human IgE *in vivo*, the signal of the human truncated mIgE expression was evident already one day after the intratumoral treatment, even if the signal of tmIgE expression was much lower compared to the signal of MVA infection.

In our future experiments, we plan to treat solid tumors induced after subcutaneous inoculation of TSA or N2C tumor cells in the "humanized" mouse model FcεRI$\alpha^{-/-}$/hFcεRIα^{+}, with intratumoral

injections of the viral vector rMVA-tmIgE. Then we will assess the response of treatment in terms of reduced tumor growth and increased survival. We expect to obtain an inflammatory reaction against the tumor (similar to that triggered during an allergic reaction) caused by the binding of IgE expressed on the surface of cells, present in the tumor mass, with FcεRI receptor expressed on the membrane of mast cells and basophils. Such IgE-dependent response would lead to a subsequent long-term protection (IgE-independent) based on a cell-mediated CD8-dependent response.

rMVA-tmIgE could be the starting point for the construction of multiple rMVA vectors expressing, in combination with human IgE, adjuvant molecules such as interleukins IL-10 and IL-15 that have already been studied in the antitumor field for their ability to increase the innate immune response (Steel JC et al. 2012) and chemokines such as CCL5 (RANTES), able to recall T cells, eosinophils, and basophils within the tumor site (Aldinucci D and Colombatti A 2014).The technology for the production of multiple rMVA was developed by our group and is already in use. We think that the results that could be obtained with this study will be a step forward towards translational studies about anti-tumor vaccination and anti-tumor immunotherapy in human.

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PAPERS ENCLOSED

In this last part of my thesis I take the liberty to enclose the title-page of the papers about studies in which planning and performing I was involved during my PhD period.

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Cutting Edge

The Journal of Immunology

Cutting Edge: IgE Plays an Active Role in Tumor Immunosurveillance in Mice

Elisa A. Nigro,* Anna T. Brini,[†,‡] Vijay A. Yenagi,[†] Lorena M. Ferreira,[†] Gertrude Achatz-Straussberger,[§] Alessandro Ambrosi,[¶] Francesca Sanvito,[∥] Elisa Soprana,* Eelco van Anken,* Gernot Achatz,[§,1] Antonio G. Siccardi,* and Luca Vangelista[#,**]

Exogenous IgE acts as an adjuvant in tumor vaccination in mice, and therefore a direct role of endogenous IgE in tumor immunosurveillance was investigated. By using genetically engineered mice, we found that IgE ablation rendered mice more susceptible to the growth of transplantable tumors. Conversely, a strengthened IgE response provided mice with partial or complete resistance to tumor growth, depending on the tumor type. By genetic crosses, we showed that IgE-mediated tumor protection was mostly lost in mice lacking FcεRI. Tumor protection was also lost after depletion of CD8+ T cells, highlighting a cross-talk between IgE and T cell–mediated tumor immunosurveillance. Our findings provide the rationale for clinical observations that relate atopy with a lower risk for developing cancer and open new avenues for the design of immunotherapeutics relevant for clinical oncology. *The Journal of Immunology,* 2016, 197: 000–000.

Immunoglobulin E is arguably the most enigmatic Ab isotype. There is clear support for an antiparasitic role of IgE (1, 2), yet most research and clinical efforts are directed at quelling IgE function as the culprit in atopic diseases, including allergy and asthma (3, 4). The pathological manifestations of IgE responses are intimately linked with the fact that IgE engages the most powerful effector arm of the immune system through activation of mast cells, basophils, and other cells (5, 6). The question is whether the antiparasitic potential of IgE fully justifies its strict conservation in the mammalian lineage, or whether IgE confers also other pivotal advantages.

Besides protection against pathogens, the immune system has an important function in tumor surveillance (7). An important branch of the immune antitumor activity is exerted by tumor-specific Abs. Several therapeutic mAbs are currently in use in oncology, and they wield a relevant portion of their clinical efficacy through Fc-mediated activation of the immune system. Ab engineering strategies that enhanced Fc-mediated antitumor activity have been implemented, including the switch from the classic IgG into the less obvious IgE framework. The redirection of the potent IgE-driven immune activation to induce tumor rejection is a strategy that has been adopted by several research groups with different approaches, either by passive or active immunotherapy (8, 9). When provided exogenously, we have previously documented that IgE is a potent adjuvant of antitumor vaccination in mice (10–12). This discovery prompted us to this work in which we explore endogenous IgE in tumor immunosurveillance, with the aim of defining a new important role for the evolution of the IgE isotype.

Materials and Methods

Mice

BALB/c mice (8 wk) were purchased from Harlan Laboratories. KN1 (13), FcεRIα knockout (KO) (14), and IgE-KO (15) mice, all in the BALB/c background, were previously described. KN1 (RRHH) males (2–4 mo) were mated with FcεRIα-KO (rrhh) females to obtain a double mutant (DM) KN1/FcεRIα-KO (rrHH). Experiments were conducted on randomized female and male mice (2–4 mo old) and were performed in accordance with institutional and state guidelines. Transgenic mice were bred in a conventional animal facility.

Genotyping

DNA was extracted by tail biopsy, and the presence of the H, h, R, and r alleles was verified by PCR using the following primers: h forward, 5′-GAAATGGACCTATAAGCTTAGAGCCTTCG-3′, h reverse, 5′-GATGTTCTTC-

*Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy; [†]Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano, 20133 Milan, Italy; [‡]IRCCS, Galeazzi Orthopedic Institute, 20161 Milan, Italy; [§]Department of Molecular Biology, Christian Doppler Laboratory for Allergy Diagnosis and Therapy, University of Salzburg, 5020 Salzburg, Austria; [¶]Vita-Salute San Raffaele University, 20132 Milan, Italy; [∥]Department of Pathology, IRCCS, San Raffaele Scientific Institute, 20132 Milan, Italy; [#]Protein Engineering and Therapeutics Unit, Division of Immunology, Transplantation, and Infectious Diseases, IRCCS, San Raffaele Scientific Institute, 20132 Milan, Italy; and **Department of Biomedical Sciences, Nazarbayev University School of Medicine, 010000 Astana, Kazakhstan

[1]Deceased.

ORCIDs: 0000-0002-5806-9495 (E.A.N.); 0000-0002-7848-8099 (A.T.B.); 0000-0002-7480-8518 (V.A.Y.); 0000-0002-4262-0406 (L.M.F.); 0000-0002-1817-7870 (G.A.-S.); 0000-0003-1976-5663 (A.A.); 0000-0001-9529-2701 (E.v.A.); 0000-0002-1654-5545 (A.G.S.); 0000-0003-0764-5031 (L.V.).

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Address correspondence and reprint requests to Dr. Luca Vangelista, Department of Biomedical Sciences, Nazarbayev University School of Medicine, Kabanbay Batyr 53, 010000 Astana, Kazakhstan. E-mail address: luca.vangelista@nu.edu.kz

Abbreviations used in this article: DM, double mutant; KO, knockout; WT, wild-type.

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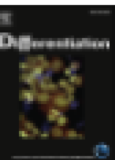

17β-estradiol differently affects osteogenic differentiation of mesenchymal stem/stromal cells from adipose tissue and bone marrow

Stefania Niada [a,b], Chiara Giannasi [a,b], Lorena Maria José Ferreira [a,b], Anna Milani [a,b], Elena Arrigoni [a,b], Anna Teresa Brini [a,b,*]

[a] Department of Biomedical, Surgical and Dental Sciences, Università degli Studi di Milano, Via Vanvitelli 32, 20129 Milan, Italy
[b] IRCCS Galeazzi Orthopaedic Institute, Via R. Galeazzi 4, 20161 Milan, Italy

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ABSTRACT

Adipose-derived and bone marrow stem/stromal cells (ASCs and BMSCs) have been often compared for their application in regenerative medicine, and several factors sustaining their differentiation and efficacy have been investigated. 17 β-estradiol (E2) has been reported to influence some functions of progenitor cells. Here we studied the effects of 10 and 100 nM E2 on ASC and BMSC vitality, proliferation and differentiation towards osteogenic and adipogenic lineages. E2 did not modulate ASC and BMSC vitality and growth rate, while the hormone produced a pro-adipogenic effect on both mesenchymal stem/stromal cells (MSCs). In particular, the synergy between 7-day pre-treatment and 100 nM E2 led to the most evident result, increasing lipid vacuoles formation in ASCs and BMSCs of +44% and +82%, respectively. Despite the fact that E2 did not alter collagen deposition of osteo-induced MSCs, we observed a different modulation of ASC and BMSC alkaline phosphatase (ALP) activity. Indeed, this osteogenic marker was always enhanced by 17 β-estradiol in BMSCs, and 7-day pre-treatment with 100 nM E2 increased it of about 70%. In contrast, E2 weakened ASC osteogenic potential, reducing their ALP activity of about 20%, with the most evident effect on ASCs isolated from pre-menopausal women (−30%).

Finally, we identified an estrogen receptor α (ERα) variant of about 37 kDa expressed in both MSCs. Interestingly, adipogenic stimuli drastically reduced its expression, while osteogenic ones mildly increased this isoform in BMSCs only.

In conclusion, E2 positively affected the adipogenic process of both MSCs while it favored osteogenic induction in BMSCs only, and both mesenchymal progenitors expressed a novel 37 kDa ER-α variant whose expression was modulated during differentiation.

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1. Introduction

Mesenchymal stem/stromal cells (MSCs) are multipotent progenitors housed in almost all vascularized adult (Crisan et al., 2008) and neonatal tissues (e.g. placenta and umbilical cord) (Hass et al., 2011). Among them, bone marrow stem/stromal cells (BMSCs) were the first to be characterized and up to now are still the most largely studied. However, adipose-derived stem/stromal cells (ASCs) represent good substitutes for clinical applications, since they are abundantly isolated from subcutaneous adipose tissue with mild donor discomfort. ASC and BMSC efficacy and safety have been largely studied, both in vitro and in vivo, and confirmed by clinical trials (Tsuji et al., 2014; Sutton and Bonfield, 2014). Nevertheless, several aspects regarding MSC isolation and culture are still under investigation and in the last years different strategies to enhance their efficiency have been explored. In particular, the identification of the ideal pool of factors required to induce MSC differentiation and to optimise their regenerative potential represents one of the most intriguing aspects to be unraveled.

In this respect, estrogens play an important role in modulating growth and differentiation of several cell types (Cooke and Naaz, 2004; Talwar et al., 2006; La Colla et al., 2015; Kovats, 2015). Among them, 17β-estradiol (E2) has been largely applied to evaluate its effects on mesenchymal progenitors; however, data are far from unequivocal (Hong et al., 2006; Leskela et al., 2006; Waters

* Corresponding author at: Department of Biomedical, Surgical and Dental Sciences, Università degli Studi di Milano, Via Vanvitelli 32, 20129 Milan, Italy.
E-mail addresses: stefania.niada@unimi.it (S. Niada), chiara.giannasi@unimi.it (C. Giannasi), lorena.ferreira@unimi.it (L.M.J. Ferreira), anna.milani@unimi.it (A. Milani), elena.arrigoni@hotmail.com (E. Arrigoni), anna.brini@unimi.it (A.T. Brini).

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Human Adipose-Derived Stem Cells on Rapid Prototyped Three-Dimensional Hydroxyapatite/Beta-Tricalcium Phosphate Scaffold

Elena Canciani, PhD, MSc, Claudia Dellavia, PhD, DDS,* Lorena Maria Ferreira, MSc,*† Chiara Giannasi, MSc,*† Daniela Carmagnola, PhD, DDS,* Antonio Carrassi, MD, DDS,* and Anna Teresa Brini, PhD, MSc*†*

Abstract: In the study, we assess a rapid prototyped scaffold composed of 30/70 hydroxyapatite (HA) and beta-tricalcium-phosphate (β-TCP) loaded with human adipose-derived stem cells (hASCs) to determine cell proliferation, differentiation toward osteogenic lineage, adhesion and penetration on/into the scaffold.

In this in vitro study, hASCs isolated from fat tissue discarded after plastic surgery were expanded, characterized, and then loaded onto the scaffold. Cells were tested for: viability assay (Alamar Blue at days 3, 7 and Live/Dead at day 32), differentiation index (alkaline phosphatase activity at day 14), scaffold adhesion (standard error of the mean analysis at days 5 and 18), and penetration (ground sections at day 32).

All the hASC populations displayed stemness markers and the ability to differentiate toward adipogenic and osteogenic lineages.

Cellular vitality increased between 3 and 7 days, and no inhibitory effect by HA/β-TCP was observed. Under osteogenic stimuli, scaffold increased alkaline phosphatase activity of +243% compared with undifferentiated samples. Human adipose-derived stem cells adhered on HA/β-TCP surface through citoplasmatic extensions that occupied the macropores and built networks among them. Human adipose derived stem cells were observed in the core of HA/β-TCP. The current combination of hASCs and HA/β-TCP scaffold provided encouraging results. If authors' data will be confirmed in preclinical models, the present engineering approach could represent an interesting tool in treating large bone defects.

Key Words: Adipose-derived mesenchymal stem cells, bone regeneration, HA/β-TCP scaffold, tissue engineering

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Langer and Vacanti[1] defined tissue engineering as a synergy between different disciplines that employs the principles of engineering to life sciences. The aim of tissue engineering is to develop biomaterials and biological substitutes able to restore, maintain, and improve tissue function. This technology is widely used in many fields such as clinical dermatology, plastic surgery, maxillo-facial surgery, and orthopedics.

The oral rehabilitation of patients, with minimum bone volume and/or unfavorable jaws relationships following periodontal disease, trauma, or oncological surgery,[2] is often achieved through techniques that include bone augmentation with homologous bone, xenografts, or synthetic biomaterials in granules or blocks.

Autologous bone is considered a gold standard graft material for its osteoinductive and osteoconductive properties. Nevertheless, only small quantities of autologous bone can be harvested and postsurgical morbidity is often an issue.[2,3]

Homologous bone, which is "fresh-frozen bone," seems to have good osteoconductive properties, but it might trigger immune responses or carry infections and long-term studies on its properties and use are still lacking.[3]

Heterologous bone (xenografts) is a graft widely used in dental practice, thanks to its osteoconductive properties and the ability to maintain its volume for a long time.[4] It acts as a scaffold for new bone formation.

Synthetic biomaterials can be used to treat large 3-dimensional (3D) bone defects. They are available in particles and blocks. The blocks can be modeled/molded to provide anatomically shaped, "custom made" scaffolds, for example by means of computer-aided design/computer-aided manufacturing technique.[5,6] It has been shown that 3D structure, surface geometry, and controlled pores with a diameter larger than 100 μm may facilitate cell colonization[7–11] and may play an important role in cell and proteins adhesion,[12] osteoblastic differentiation,[5] and vascular colonization.[10,13]

In the last years stem cells have been considered to be good candidates for improving the performance of different biomaterials in tissue regeneration and many studies about stem cells combined with 3D scaffolds have shown promising results in bone regeneration both in vitro and in vivo.[5,9,14–16]

Different types of mesenchymal stem cells (MSCs) have been evaluated in vitro[17,18] and in animal models, for the treatment of large bone defects,[19,20] combined with growth factors like BMP-2[5] or loaded on various biomaterials including 3D scaffolds.[5,9,14,19]

Bone marrow is the most common source used for MSCs isolation but it has some limitations: the invasiveness of the withdrawal, postsurgical pain, and low cell yield.[21] MSCs obtained from subcutaneous adipose tissue have been suggested as an alternative source, since human adipose stem cells (hASCs) are collectable through liposuction under local anesthesia and adipose tissue allows to obtain a large number of cells.[22–24]

From the *Department of Biomedical, Surgical and Dental, Università degli Studi di Milano; and †IRCCS Galeazzi Orthopaedic Institute, Milan, Italy.
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Address correspondence and reprint requests to Dr Elena Canciani, PhD, MSc, Department of Biomedical, Surgical and Dental, Università degli Studi di Milano, Via Mangiagalli 31, 20133 Milan, Italy;
E-mail: elena.canciani.mi@gmail.com
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ORIGINAL RESEARCH

Cell-mediated drug delivery by gingival interdental papilla mesenchymal stromal cells (GinPa-MSCs) loaded with paclitaxel

Anna Teresa Brini [a,b,*], Valentina Coccè [a,*], Lorena M. Josè Ferreira [a], Chiara Giannasi [a], Gianguido Cossellu [c], Aldo Bruno Giannì [d], Francesca Angiero [e], Arianna Bonomi [a], Luisa Pascucci [f], Maria Laura Falchetti [g], Emilio Ciusani [h], Gianpietro Bondiolotti [i], Francesca Sisto [a], Giulio Alessandri [j], Augusto Pessina [a,†] and Giampietro Farronato [a,c,†]

[a]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; [b]I.R.C.C.S. Istituto Ortopedico Galeazzi, Milan, Italy; [c]Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Unit of Orthodontics and Paediatric Dentistry, School of Dentistry, Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; [d]Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Maxillofacial and Dental Unit, Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; [e]Department of Medical Sciences and Diagnostic Integrated, S. Martino Hospital, University of Genoa, Genoa Italy; [f]Department of Veterinary Medicine, University of Perugia, Perugia, Italy; [g]CNR – Institute of Cell Biology and Neurobiology, Rome, Italy; [h]Laboratory of Clinical Pathology and Neurogenetic Medicine, Fondazione IRCCS Neurological Institute Carlo Besta, Milan, Italy; [i]Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy; [j]Cellular Neurobiology Laboratory, Department of Cerebrovascular Diseases, IRCCS Neurological Institute C. Besta, Milan, Italy

ABSTRACT
Objective: Gingival tissue is composed of cell types that contribute to the body's defense against many agents in oral environment, wound healing and tissue regeneration. Thanks to their easy and scarcely invasive withdrawal procedure, interdental papilla provide a good source of mesenchymal stromal cells (GinPa-MSCs). We isolated GinPa-MSCs and verified their ability to uptake/release the anticancer agent Paclitaxel (PTX).
Methods: *In vitro* expanded GinPa-MSCs were characterized for CD markers by FACS, tested for differentiation ability and analyzed by TEM. Their ability to uptake/release PTX was assessed according to a standardized procedure.
Results: The CD expression and chondro-adipo-osteo differentiation ability confirmed the mesenchymal feature of GinPa-MSCs. Surprisingly, 28% of GinPa-MSCs expressed CD14 marker and had an impressive pinocytotic activity. GinPa-MSCs were able to take up and release a sufficient amount of PTX to demonstrate effective *in vitro* activity against pancreatic carcinoma cells, suggesting that the drug was not inactivated.
Conclusions: The procedure to obtain MSCs from interdental papilla is less invasive than that used for both bone marrow and adipose tissue, GinPa-MSCs are easy to expand and can be efficiently loaded with PTX. Taken together these qualities suggest that GinPa-MSCs may prove to be a good tool for cell-mediated drug delivery in cancer, particularly if related to stomatognathic system.

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1. Introduction

In the past years, mesenchymal stromal cells (MSCs) have been isolated from several dental sources, such as dental pulp,[1] exfoliated deciduous teeth,[2] apical papilla,[3] dental follicle,[4] and gingival margin.[5] This matter has been reviewed by Huang et al. [6] and recent studies further indicate that the gingivae contain both neural crest- and mesoderm-derived MSCs.[7]

Considering the tissue origin, the gingival margin represents the main component of the periodontium. Its development shows the contribution from different structures: the perifollicular mesenchyme,[8] the inner layer of the dental follicle,[9] the periodontal ligament and their resident stem/progenitor cells.[10,11] However, there are several differences among these sources concerning accessibility and availability of stem cells. Among oral MSCs, gingival MSCs can be easily isolated since this tissue is routinely removed during dental crown lengthening and periodontal surgical procedures. As recently reported by Fawzy El-Sayed et al.,[12] the gingival margin represents a good source of multipotent stem/progenitor cells, both in terms of cellular yield and stemness features. However, according to some authors,[13] gingival MSCs have a relatively lower osteogenic differentiation ability than those isolated from other tissues. Furthermore, gingival MSCs display rapid wound healing properties that allow tissue repair without producing significant scarring.[14] Cells with

CONTACT Augusto Pessina augusto.pessina@unimi.it Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via Pascal 36, 20133 Milan, Italy

*These authors contributed equally to this work.
†Shared senior's authorship.
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Assessment of autologous and allogeneic equine adipose-derived stem cell therapeutic use in musculo-skeletal tissues diseases: a pilot study

Valutazione dell'utilizzo terapeutico di cellule staminali equine autologhe e allogeniche derivate da tessuto adiposo per patologie a carico di tessuti muscolo-scheletrici: uno studio pilota

Lorena Maria José Ferreira*[1,2],
Stefania Niada*[1,2],
Fabrizio Monici[3],
Alessandro Garbieri[3],
Chiara Giannasi[1],
Anna Teresa Brini[1,2]

[1]Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano, Milano
[2]IRCCS Istituto Ortopedico Galeazzi, Milano
[3]Libero professionista
*Contributo equivalente

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Parole chiave
Cellule staminali derivate da tessuto adiposo, terapia cellulare, lesioni muscolo-scheletriche, medicina equina.

Corrispondenza
Stefania Niada,
Dipartimento di Scienze Biomediche,
Chirurgiche e Odontoiatriche,
Università degli Studi di Milano,
Via Vanvitelli 32,
20129 Milano.
E-mail: stefania.niada@unimi.it

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SUMMARY / The aim of regenerative medicine is to develop new strategies to restore damaged tissues. Stem cells, due to their ability of self-renewal and differentiation, might play a role in this growing field. Mesenchymal stem cells are multipotent cell derived from adult tissues with peculiar features which can be exploited for future cellular therapy. Here we investigated the effect of injected autologous and allogenenic adipose-derived stem cells (ASCs) from 8 adult injured horses. Equine adipose-derived stem cells (eqASCs) proliferate fast (mean duplication time=62.3±23.5) allowing to rapidly collect a large number of cells; they display classical stemness features, such as clonogenic (12.8±5.1%) and differentiative ability. We inoculated 10 horses (12.8±2.4 y/o) at the level of injured tendon, ligament and articular cartilage, with at least $5x10^6$ eqASCs. After 4 months, 9/10 horses showed a good clinical outcome with lameness reduction, and 8/10 horses restarted activity. At longer follow-up (8-21 months), one of the 8 horses in activity showed an additional amelioration, while one relapsed. This pilot study suggests that in vitro expanded (cryopreserved or not), eqASCs exert a therapeutic effect on musculo-skeletal injuries, probably also related to their anti-inflammatory action. In conclusion, like human lipoaspirate, equine adipose tissue contains multipotent cells useful for veterinary cell therapy as well as preclinical studies.

RIASSUNTO / L'obiettivo della medicina rigenerativa è quello di sviluppare nuove strategie per la riparazione di tessuti danneggiati e le cellule staminali sembrano avere un ruolo importante in questo campo. In particolare, le cellule staminali mesenchimali sono cellule multipotenti isolabili da tessuti adulti e posiedono caratteristiche appropriate per la terapia cellulare. Un numero elevato di cellule staminali mesenchimali, derivate da tessuto adiposo di 8 cavalli (eqASC), è stato ottenuto in tempi brevi; esse posiedono le tipiche caratteristiche di staminalità quali capacità proliferativa (tempo medio di duplicazione=62,3±23,5), clonogenica (12,8±5,1%) e differenziativa. $5x10^6$ di eqASC, autologhe o allogeniche, sono state inoculate nella sede di tessuti lesionati, quali tendini, legamenti e cartilagine articolare, in 10 cavalli (età 12,8±24 anni). Al primo follow-up (4 mesi) si è osservata una riduzione variabile della zoppia in 9 cavalli su 10 (2 un lento miglioramento, 4 un buon miglioramento, 3 una remissione totale della zoppia); uno non ha avuto alcun beneficio e 8 cavalli hanno ripreso l'attività. Al follow-up successivo (8-21 mesi) il quadro clinico per 8 cavalli si è mantenuto, un soggetto in attività ha mostrato un ulteriore miglioramento, mentre un altro una recidiva. Questo studio pilota suggerisce che le eqASC espanse in vitro, anche dopo crio-preservazione, hanno effetti clinici benefici su diverse lesioni muscolo-scheletriche probabilmente dovuti anche ad un'attività antinfiammatoria delle ASC. In conclusione, il tessuto adiposo equino contiene cellule multipotenti utili per la terapia cellulare veterinaria e per studi preclinici.