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An Antitumor Cellular Vaccine Based on a Mini-Membrane IgE

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The IgE-mediated immune system activation can be redirected to combat tumors. Mouse and human IgE have been shown to provide a potent adjuvant effect in antitumor vaccination, with a crucial role played by FcεRI. This effect results from T cell-mediated adaptive immune response. Modified vaccinia virus Ankara (MVA) has been used to infect IgE-loaded tumor cells. These results led to a shift toward a highly safe protocol employing membrane IgE (mIgE), thus eliminating any possible anaphylactogenicity caused by circulating IgE. Evidence that human mIgE and a truncated version lacking IgE Fabs (tmIgE) bind and activate FcεRI has been fundamental and forms the core of this report. Human tmIgE has been engineered into a recombinant MVA (rMVA-tmIgE), and the expression of tmIgE and its transport to the surface of rMVA-tmIgE-infected cells has been detected by Western blot and cytofluorimetry, respectively. FcεRI activation by tmIgE has been confirmed by the release of β-hexosaminidase in a cell-to-cell contact assay using human FcεRI-transfected RBL-SX38 cells. The rMVA-tmIgE antitumor vaccination strategy has been investigated in FcεRIα^{-/-} human FcεRIα⁺ mice, with results indicating a level of protection comparable to that obtained using soluble human IgE tumor cell loading. The rMVA-tmIgE vector represents a device that suits safe IgE-based antitumor vaccines, harboring the possibility to couple tmIgE with other gene insertions that might enhance the antitumor effect, thus bringing the field closer to the clinics. *The Journal of Immunology*, 2012, 188: 000–000.

The power of the IgE–FcεRI interaction resides in its high affinity and highly coordinated binding mode that, upon appropriate circumstances, promote receptor activation, resulting in the well-known deleterious manifestations and less-defined beneficial effects (1–3). The long-sought solution to the quest on the physiologic role IgE should play within the immune system is still awaited. However, the resurgence of the IgE system during the evolution of mammal immunity is likely to go beyond the protection against intestinal parasites, with allergic conditions as a sometime dramatic example of the dysregulation of the system. In the search for its comprehensive immune identity, an intriguing yet hypothetical scenario sees IgE as an antitumor surveillant (4, 5). To date, controversial epidemiological studies and scattered clinical evidence do not allow a conclusion on

whether this hypothesis holds true (6, 7). A few research groups approached the question using different experimental setups, all based on the final docking of IgE on its receptors (6, 7). Among these attempts, one of special interest might be represented by the possibility to isolate and identify tumor-specific monoclonal IgE from individuals that survived cancer (8). Once these monoclonal IgE Abs could be characterized, they might reveal powerful antitumor features. Initial redirection of the IgE system toward cancer was attempted by various groups working on engineered anti-tumor-associated Ag (TAA) monoclonal IgE Abs that proved to be powerful elicitors of antitumor responses (reviewed in Refs. 6, 7). Following this evidence, a different approach has been undertaken in which attempts of raising antitumor endogenous IgE are being investigated (reviewed in Refs. 6, 7, and E.A. Nigro, A.G. Siccardi, and L. Vangelista, unpublished observations).

Another effective way to combat cancer with IgE could be embodied in its use as adjuvant in antitumor vaccination, making use of the whole IgE–FcεRI-driven machinery to activate the host immune system. Through different in vitro and in vivo tumor cell-targeting strategies, tumor cell lines, genetically modified mice, and the use of mouse as well as human IgE, previous reports in this direction proved IgE antitumor adjuvant activity and the dominant role played by the IgE–FcεRI interaction in the establishment of the adjuvant effect (6, 7, 9, 10). This approach coupled tumor cell targeting by IgE with the activation of the host immune system to efficiently help in establishing an antitumor immune memory, resulting from priming of the T cell-mediated adaptive immune response (9). The protective effect should be attributed to the interaction of tumor cell-bound IgE with FcεRI on the surface of mast cells and basophils, which, upon FcεRI activation, undergo a massive release of mediators by degranulation, that in turn promote recruitment of effector cells, tumor cell killing, and finally tumor Ag cross-priming (6, 7).

The present work represents the molecular evolution of an IgE-based antitumor vaccine implementing modified vaccinia virus

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Abbreviations used in this article: CEF, chicken embryo fibroblasts; EGFP, enhanced GFP; h, human; LACK, *Leishmania* receptor for activated C kinase; mIgE, membrane IgE; MVA, modified vaccinia virus Ankara; NC, negative control; NIP, 4-hydroxy-5-iodo-3-nitrophenylacetyl; rMVA, recombinant modified vaccinia virus Ankara; scFc, single-chain Fc; TAA, tumor-associated Ag; tmIgE, truncated membrane IgE; TPG, transfer plasmid Green; VV, vaccinia virus.

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(VV) Ankara (MVA) for the infection and subsequent killing of vaccinating tumor cells in a strategy stemming from the capacity of human membrane IgE (mIgE) to activate FcεRI in an Ag-independent manner (11). The cDNA of a human truncated mIgE (tmIgE), yet competent for FcεRI activation (11), was inserted into a recombinant MVA (rMVA) using a recently reported, reliable rMVA production technology (12, 13). The resulting rMVA-tmIgE constitutes a compact and safe molecular device that assures tumor cell infection and death with the simultaneous exposure of tmIgE on the cell surface. The presence of circulating IgE is thus eliminated, yet providing an alternative molecular route for the IgE–FcεRI interaction, instrumental for the establishment of the IgE antitumor adjuvanticity. Moreover, human IgE and the compact rMVA-tmIgE system (not effective in wild-type BALB/c mice) had a protective effect in the FcεRIα^{−/−} human (h)FcεRIα⁺ BALB/c mouse (10, 14), suggesting that the adjuvant effect of IgE could be exploited for human vaccinology.

Materials and Methods

Mice

Experiments conducted on mice were performed in accordance with institutional and state guidelines. BALB/c mice (female 8 wk of age) were purchased from Harlan Laboratories. FcεRIα^{−/−}hFcεRIα⁺ and FcεRIα^{−/−} (BALB/c background) mice were previously described (14, 15). Transgenic mice were bred in a conventional animal facility.

Cell lines

TS/A-*Leishmania* receptor for activated C kinase (LACK) is an H-2^d mammary adenocarcinoma of BALB/c origin, kindly provided by A. Mondino (San Raffaele Scientific Institute, Milan, Italy), maintained in RPMI 1640 medium (Life Technologies Invitrogen), and supplemented with 100 μg/ml geneticin (G418) (Calbiochem). RBL-SX38 is an RBL-2H3-derived (rat basophilic leukemia) cell line expressing the α, β, and γ-chain of human FcεRI (16) maintained in DMEM medium (Life Technologies) and 800 μg/ml G418. All cultures were supplemented with 100 U/ml penicillin/streptomycin (Life Technologies) and 10% FCS (Euroclone). Fresh chicken embryo fibroblasts (CEF) were prepared from 11-d-old embryonated eggs from SPF chicken and maintained in serum-free medium containing 10 ng/ml epidermal growth factor (VP-SFM; Life Technologies) supplemented with glutamine and antibiotics. Fresh CEF could also be frozen in Serum-Free Cell Freezing Medium (Biological Industries) before being cultured in VP-SFM. Fresh CEF could be passaged three to four times, frozen CEF only one to two times. Serum-free CEF yielded MVA titers of ~10⁷ PFU/ml after 48 h of culture, very similar to the titers produced by serum-supplemented cultures, as already reported (17). Serum-free CEF were used for recombinant virus construction, terminal dilution cloning, and virus stock production, whereas for analytical experiments, DMEM supplemented with 5% FCS, 1% chicken serum (Life Technologies), and antibiotics was used. The derivation of MVA-Red from MVA (Innew) (18) was already described (12).

Virus and virus construction

MKG is an MVA with enhanced GFP (EGFP) in deletion VI (a kind gift of V. Erfle, Munich Technical University, Munich, Germany), named hereafter as MVA-Green for simplicity. The production of rMVA-tmIgE was preceded by a series of plasmid constructions and an intermediate quality check of tmIgE expression. The pcDNA3 construct encoding for tmIgE (11) was HindIII/KpnI digested, followed by ApaLI digestion, to subclone the ApaLI/KpnI 1394-bp fragment into a modified pUT-SEC vector (19) to substitute the genomic mouse Ig secretion leader, containing an intron, with an intronless leader sequence, necessary for rMVA-induced protein expression, as similarly reported for a mouse tmIgE construct (12). A HindIII/KpnI 1455 fragment was then excised from the intronless pUT-SEC-tmIgE construct and ligated into HindIII/KpnI-linearized pcDNA3 to obtain an intronless pcDNA3-tmIgE construct. To assure proper tmIgE expression after the leader sequence modification, 293T cells were transiently transfected with intronless pcDNA3-tmIgE. Cell-surface expression of tmIgE was confirmed by cytofluorimetry using goat anti-human IgE FITC-conjugated polyclonal Abs (KPL) (data not shown), and the ability of tmIgE to activate human FcεRI was confirmed by RBL-SX38 cells β-hexosaminidase release (data not shown). Following, intronless pcDNA3-tmIgE was HindIII-linearized and blunted by Klenow DNA

polymerase I (New England Biolabs) and subsequently subjected to EcoRV digestion. The blunted 1510-bp resulting fragment was inserted into a PmeI-linearized transfer plasmid Green (TPG) encoding for EGFP (13) to obtain the final TPG-tmIgE construct. Correct orientation of the tmIgE cDNA insert was monitored by single BamHI and KpnI digestions. Fast production of rMVA-tmIgE followed the described Red-to-Green gene swapping methods (13). Briefly, CEF were subjected to infection/transfection in six-well culture plates using MVA-Red, encoding for the red fluorescent protein HcRed1-1 (12, 13), at a multiplicity of infection of 0.2 and TPG-tmIgE (10 μg), respectively. Fluorescence microscopy of living infected cells was performed using an inverted Olympus IMT-2 microscope (Olympus Optical, Milan, Italy) and images taken by a digital camera (Olympus CAMEDIA-C2000). Virus-containing cell lysates (from the infection/transfection) were used (at a 1:10 dilution) to infect semiconfluent CEF in six-well culture plates. Two hours postinfection, the cultures were washed and supplemented with fresh medium containing 1 μM cytochalasin D (Sigma-Aldrich) to reversibly inhibit virus release and thus superinfection (13). Twenty-four hours postinfection, cells were trypsinized, resuspended, and single-cell sorted by a Becton Dickinson FACS Vantage SE flow cytometer (Milan, Italy). EGFP fluorescence (excitation at 488 nm) was detected using a 530/30-nm bandpass filter. Single-cell sorting generated a terminal dilution 96-well plates for which monitoring was performed by whole-plate Typhoon fluoroimaging, which allows visualization of green or red fluorescence in microcultures. Six rMVA-tmIgE clones were identified that emitted exclusively green fluorescence and were further characterized for tmIgE expression.

Western blot

Cells (10⁶) were infected at a multiplicity of infection of 1 (CEF) and 10 (TS/A-LACK) with rMVA-tmIgE, MVA, or were not infected. CEF and TS/A-LACK cells were lysed 48 h postinfection in TNN buffer (50 mM Tris-HCl [pH 8], 250 mM NaCl, and 0.5% Nonidet P-40) containing protease inhibitors (200 μg/ml PMSF; Sigma-Aldrich; and a protease inhibitor mixture, Complete; Roche) and 20 mM alkylating agent *N*-ethylmaleimide (Sigma-Aldrich) for 30 min at 4°C. Cell lysates were centrifuged at 4°C for 10 min at 10,000 × *g*, and protein samples were loaded under reducing or nonreducing conditions onto 10% SDS-PAGE and transferred to nitrocellulose (Hybond ECL; Amersham Biosciences). tmIgE was detected with HRP-conjugated anti-human IgE Abs (Dako-Cytomation). Western blots were developed by ECL (Amersham Biosciences) autoradiography.

IgE loading of tumor cells by haptenization

Human IgE loading was carried out incubating tumor cells with 4-hydroxy-5-iodo-3-nitrophenylacetyl (NIP)-hydroxysuccinimidyl ester (Cambridge Research Biochemicals), 0.2 mg/10⁶ cells in 10 mM sodium phosphate (pH 7.2) (10⁶ cells/ml) for 45 min at room temperature. Following a 10-min blocking reaction with RPMI 1640 medium supplemented with 10% FCS and washing, cells were incubated 45 min at 4°C in PBS containing 5% BSA with 20 μg/ml purified chimeric anti-NIP human IgE mAb secreted by a stably transfected J558L cell clone, as previously reported (11).

Cytofluorimetry

Cellular IgE loading or tmIgE cell-surface expression was monitored by cytofluorimetry incubating 5 × 10⁶ tumor cells with 5 μg/ml biotinylated mouse anti-human IgE mAb (BD Pharmingen) in PBS containing 5% BSA for 30 min at 4°C. After washing, cells were incubated with PE-conjugated streptavidin (BD Pharmingen) in PBS containing 5% BSA for 20 min at 4°C and fixed with PBS containing 0.4% paraformaldehyde. Cytofluorimetry was performed on a FACSCalibur (BD Biosciences), and CellQuest software (BD Biosciences) was used for sample acquisition and data analysis.

Mediator release

Similar to previously reported methods (10, 11), plastic-adherent RBL-SX38 cells (8 × 10⁴ cells/well) were incubated in DMEM with 100 ng chimeric anti-NIP human IgE mAb (described above) for 2 h at 37°C. Cells were then washed and incubated in triggering buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM CaCl₂, 0.4 mM MgCl₂, and 0.1% BSA [pH 7.2]) with 100 ng NIP-BSA (Biosearch Technologies) for 1 h at 37°C. Alternatively, IgE-loaded, tmIgE-expressing, or IgE-free tumor cells in triggering buffer were added to RBL-SX38 cells (at a 2:1 ratio). Plates were centrifuged 5 min at 300 × *g* and incubated for 1 h at 37°C. The release of β-hexosaminidase by RBL-SX38 cells was detected in the cell supernatant. Supernatants were transferred to a new plate, and 1 mM p-nitrophenyl-*N*-acetyl-β-D-glucosamide (Sigma-Aldrich) in 0.1 M citrate buffer (pH 6.2) was added, followed by a 150-min incubation at 37°C. The

reaction was stopped using 0.1 M stop solution (0.1 M Na_2CO_3 and 0.1 M NaHCO_3 [pH 10]), and the absorbance was read at 405 nm. Negative control (NC) was the supernatant of nonstimulated cells. Positive control was the supernatant of human IgE-sensitized RBL-SX38 cells stimulated with 100 ng NIP-BSA. The results are calculated as percentage of the positive control using $100 \times (A_{405} \text{ sample} - A_{405} \text{ NC}) / (A_{405} \text{ positive control} - A_{405} \text{ NC})$.

Vaccination experiments

Immunizations were performed on five mice for each group by s.c. administration of rMVA-tmIgE-infected or MVA-infected IgE-loaded TS/A-LACK cells (10^5 cells/mouse) in PBS at the base of the tail. MVA-infected tumor cells were used as a control. Two weeks after the immunization, mice were challenged by s.c. injection in the left flank with 2×10^5 TS/A-LACK cells in PBS. Tumor growth was monitored at 1–3-d intervals by measuring tumor volume with a caliper. Tumor volume, V , was calculated by the equation $V = 4/3\pi \times r_1 \times r_2 \times r_3$, in which r_1 is the longitudinal radius, r_2 is the lateral radius, and r_3 is the thickness of tumors protruding from the surface of normal skin (10, 20).

Statistics

The statistic model used for the analysis of tumor growth is that reported previously (10). In each set of experiments, we assume that the tumor growth follows an exponential pattern as function of time. More specifically, we assume that $v_{ijt} = \alpha_i e^{\beta_{it}} \varepsilon_{ijt}$, in which v_{ijt} is the tumor volume measured in the i th experimental line in the j th mice at time t . The parameters β_i are fixed-effect coefficients that give the measure of tumor volume progression; α_i are random-effect coefficients for group i , and they are assumed to be independent lognormally distributed. These parameters are thought as random variables and describe unwilld possibly random differences in initial conditions at time 0 between groups; ε_{ijt} is the observation error for measurement made at time t on j th mice of the i th experimental line. Errors ε_{ijt} are assumed to have a multivariate lognormal distribution. Mixed random-fixed effects models (21) are successfully applied in a number of studies (22–26). Because observations represent longitudinal data on single individuals, we considered a first-order autocorrelation structure among errors. Furthermore, errors in our model are assumed to have multiplicative effect on observed measurements. Such assumptions guarantee that observed measurements are always positive with variance monotonically increasing with the tumor volume, which is empirically observed. The hypothesis tested concerned differences between progressions ($H_0: \beta_i = \beta_{i'}, H_1: \beta_i \neq \beta_{i'}, \forall i \neq i'$). It can be shown (21) that these testing procedures lead to t -type tests. Statistical significance was defined as a value of $p < 0.05$.

Results

Membrane IgE as antitumor adjuvant

In previous studies (7, 9), we demonstrated that soluble mouse IgE loaded onto tumor cells presents an antitumor adjuvant effect in cellular vaccines in vivo. IgE adjuvanticity could be exploited for different tumors, and it did not depend on specific TAA expression. Moreover, this effect has been confirmed also for human IgE, and the crucial role FcεRI plays in this scenario has been demonstrated (7, 10). Next, a membrane-bound version of human IgE was implemented by engineering it into an rMVA to provide the mean for both vaccinating tumor cell killing and tmIgE expression (Fig. 1).

The tmIgE construct is a truncated version of human membrane IgE isoform long that encodes for the Cε3 and Cε4 domains, the extracellular membrane-proximal domain, the transmembrane, and cytoplasmic moieties and carries a SV5 tag at its N terminus (11). These molecular features provide easy detection, membrane anchorage, the necessary information for appropriate folding and dimerization, cell-surface exposure, and, ultimately, FcεRI recognition, all in a single polypeptide chain (11) (Fig. 1). Intermolecular tmIgE disulphide bonds occur via cysteine residues within the extracellular membrane-proximal domain (27) and the N-terminal Cε3 domain cysteine residue (C328) undergoing homodimerization in absence of the Cε2 domain, yet engaging with a Cε2 cysteine residue (C241) in complete IgE (2, 28, 29). C328 differential disulphide bonding mode (i.e., in presence or absence

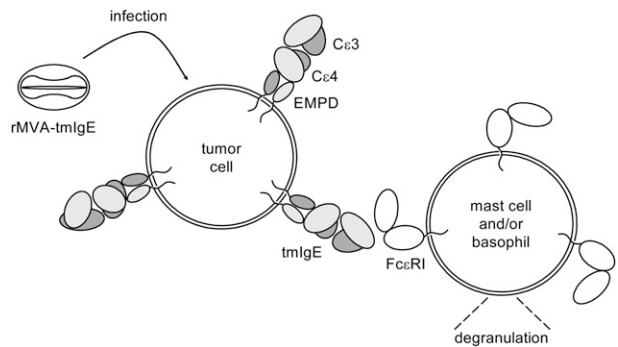


FIGURE 1. Schematics of the cellular antitumor vaccine based on membrane IgE. Infection of tumor cells with rMVA-tmIgE warrants expression of tmIgE and, subsequently, vaccinating tumor cells' death. The single polypeptide chain format of tmIgE possesses all of the necessary information for folding, dimerization, and transport to the cell surface, crucial requisites to assure FcεRI recognition and activation. Once activated, FcεRI⁺ mast cells and basophils undergo massive degranulation, releasing the classic mediators of allergic reactions. The IgE–FcεRI interaction is the initial event leading to IgE antitumor adjuvanticity. Only the α-chain of FcεRI is represented in the scheme, and the N-terminal SV5 tag of tmIgE is not reported. EMPD, extracellular membrane-proximal domain.

of the Cε2 domain) does not affect FcεRI binding and, in engineered variants, such as tmIgE or soluble Cε3–Cε4, homodimerization of C328 provides stabilization of the protein (11, 28). Recent structural data on a Cε2–Cε3–Cε4 construct in complex with FcεRI α-chain D1–D2 domains (30) evidenced that the Cε2 domain provides the basis for the overall affinity of the IgE–FcεRI interaction, mainly adding to the unusually slow dissociation rate, not present in Cε3–Cε4 constructs. Yet, the Cε3–Cε4 format efficiently binds to FcεRI and competes with IgE for FcεRI binding. The reported ability by human mIgE (confirmed also for tmIgE) to reach the cellular surface also in absence of the BCR accessory proteins Igα and Igβ (11) represents a further advantage for the design of a membrane-anchored antitumor cellular vaccine based on IgE (Fig. 1).

Construction and preparation of rMVA-tmIgE and in vitro characterization of tmIgE

Altogether, tmIgE molecular features provided the ideal IgE single-chain format for our purposes. Thus, the original tmIgE-encoding pcDNA3 construct (11) was modified to eliminate the intron present in the mouse Ig secretion leader genomic DNA, obtaining an intronless pcDNA3-tmIgE used to verify tmIgE appropriate expression and function by transient transfection of 293T cells (data not shown). The tmIgE cDNA was then subcloned into TPG, an EGFP-encoding vector that allowed the engineering of rMVA-tmIgE via the Red-to-Green gene swapping technology (Fig. 2A) (12, 13). CEF infection/transfection with MVA-Red/TPG-tmIgE led to recombinant Green viruses, detected by fluorescence microscopy (Fig. 2B). CEF were used because they are accepted and recognized as good manufacturing practice, therefore virus production in CEF represents a high-quality/safety production procedure. A cell lysate from CEF infection/transfection was used to infect semiconfluent CEF that were then subjected to single-cell sorting by flow cytometry. After terminal dilution culture, six CEF-derived rMVA-tmIgE clones have been selected for tmIgE characterization. Expression of tmIgE by these rMVA-tmIgE clones has been assessed by a previously reported (13) immunoperoxidase staining assay (data not shown) and Western blot analysis under reducing conditions on total CEF extracts, with the detection of the expected major band at ~40 kDa (11) (Fig. 2C). Transport to the cell surface of tmIgE has been detected by

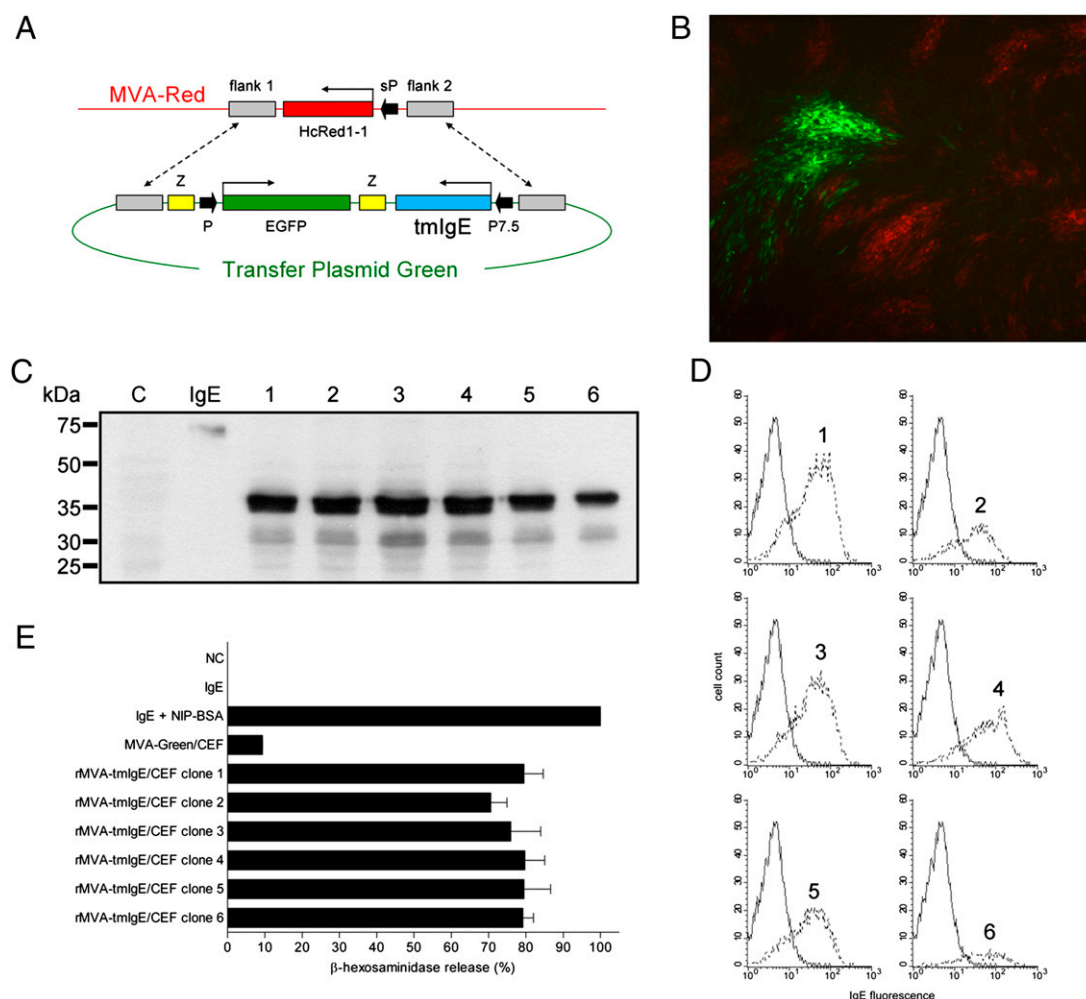


FIGURE 2. Construction of rMVA-tmIgE and clone selection. **A**, Genetic scheme of the infection/transfection reagents for the Red-to-Green technology (12, 13). MVA-Red encodes for the red fluorescent protein HcRed1-1 (under the sP promoter) flanked by two regions for homologous recombination (flanks 1 and 2). TPG encodes for an expression cassette flanked by homologous recombination regions identical to those in MVA-Red (flanks 1 and 2). The TPG cassette is composed of EGFP cDNA (under the sP promoter, P) flanked by two identical Z regions that allow the spontaneous excision of EGFP to obtain colorless rMVA as a final product. Following that, the cassette contains the transgene's cDNA (tmIgE) under the P7.5 promoter. **B**, Fluorescence microscopy of infected/transfected CEF, a rare field containing both red and green clones. Red cells are infected but nontransfected; green cells are infected and transfected. Original magnification $\times 50$. **C**, Total cell extract Western blot analysis of CEF infected with rMVA-tmIgE clones (1–6). As an NC, CEF were infected with MVA-Green (C), and human soluble anti-NIP IgE was used as positive control (the band at ~ 75 kDa is IgE H chain). Samples were analyzed in reducing conditions using HRP-conjugated anti-human IgE. **D**, Cytofluorimetric analysis of CEF-infected with rMVA-tmIgE clones (1–6). Cell-surface expression of tmIgE was detected using biotinylated anti-human IgE followed by streptavidin-PE. As control (C), MVA-Green-infected CEF were analyzed. **E**, rMVA-tmIgE clone-mediated β -hexosaminidase release assay. RBL-SX38 cells were put in contact with MVA-Green-infected (MVA-Green/CEF) or rMVA-tmIgE clone-infected (rMVA-tmIgE/CEF clones 1–6) CEF. The 100% release positive control consists of soluble anti-NIP IgE-sensitized RBL-SX38 cells cross-linked with NIP-BSA (IgE + NIP-BSA). NC, nonstimulated RBL-SX38 cells; IgE, human soluble anti-NIP IgE-loaded RBL-SX38 cells in the absence of NIP-BSA. Values are mean $\pm$ SD of three determinations.

cytofluorimetry (Fig. 2D). Most importantly, rMVA-tmIgE clones have been tested for the capacity by tmIgE to activate Fc ϵ RI using the cell–cell β -hexosaminidase release assay on RBL-SX38 cells (Fig. 2E) (10, 11). As already reported for its stable transfection on sp2/0 cells (11), tmIgE proved to be fully functional also when encoded by rMVA upon infection of CEF, validating the rationale behind the use of rMVA-tmIgE in antitumor vaccination. The six clones presented similar features; hence, rMVA-tmIgE clone 1 has been selected, used throughout all successive experiments, and named rMVA-tmIgE for simplicity.

In vitro characterization of the rMVA-tmIgE/TS/A-LACK antitumor cellular vaccine

Following rMVA-tmIgE production and characterization in CEF, the efficacy of the rMVA-tmIgE strategy has been tested in vitro on

TS/A-LACK cells, assessing expression and assembly of tmIgE as the expected covalent dimer (Fig. 3A), its surface exposure (Fig. 3B), and its ability to induce RBL-SX38 cell β -hexosaminidase release upon cell–cell contact (Fig. 3C). The appropriate rMVA-embedded tmIgE function confirms MVA as a safe vehicle for vaccinating cell killing (10) coupled to the possibility of adjuvant (tmIgE) delivery to the cell membrane. The rMVA-tmIgE infection of TS/A-LACK cells was therefore ready to be tested in vivo as antitumor cellular vaccine using the appropriate mouse strain. The Fc ϵ RI $\alpha^{-/-}$ hFc ϵ RI α^{+} BALB/c mouse (14) was therefore used, as it already proved convenient to test the antitumor adjuvanticity of human IgE (10). Samples used for this in vitro characterization are aliquots of preparations actually used for the in vivo experiments, thus reflecting the quality of injected material.

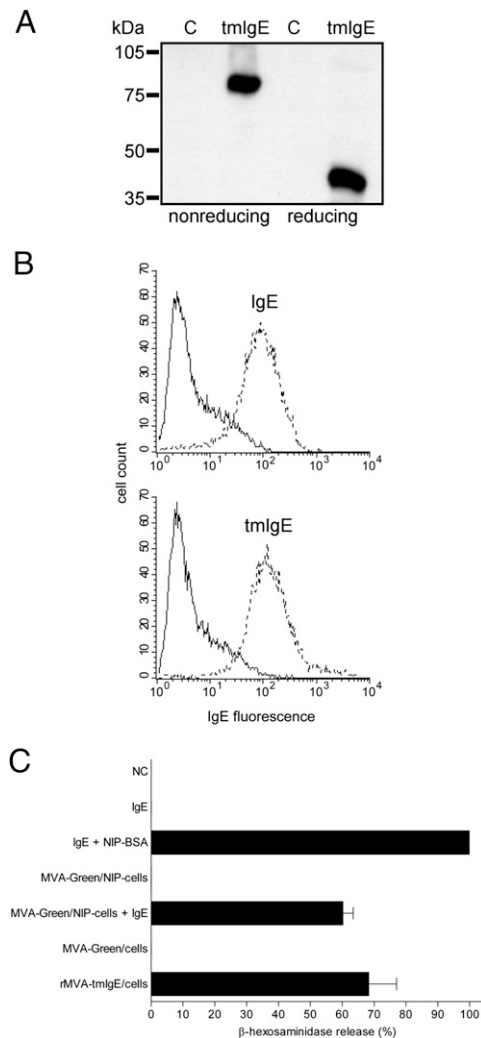


FIGURE 3. In vitro characterization of the rMVA-tmIgE/TS/A-LACK antitumor cellular vaccine. *A*, Total cell extract Western blot analysis of TS/A-LACK cells infected with rMVA-tmIgE (tmIgE). As an NC, TS/A-LACK cells were infected with MVA-Green (C). Samples were analyzed both in reducing and nonreducing conditions using HRP-conjugated anti-human IgE, and the expected (11) tmIgE bands at ~40 and ~80 kDa were revealed, respectively. *B*, Cytofluorimetric analysis of antitumor cellular vaccines. *Top panel*, MVA-Green-infected tumor cells were NIP-haptenized and loaded with human anti-NIP IgE (dotted line) or not loaded (continuous line). *Bottom panel*, MVA-Green-infected TS/A-LACK cells (continuous line) or TS/A-LACK cells infected with rMVA-tmIgE (dotted line). IgE loading (IgE) or cell-surface expression (tmIgE) was verified by cytofluorimetric analysis using biotinylated anti-human IgE followed by streptavidin-PE. *C*, IgE-mediated β -hexosaminidase release assay. RBL-SX38 cells were put in contact with MVA-Green-infected, IgE-loaded (MVA-Green/NIP-cells + IgE) or not loaded (MVA-Green/NIP-cells) haptenized tumor cells. Alternatively, RBL-SX38 cells were put in contact with MVA-Green-infected (MVA-Green/cells) or rMVA-tmIgE-infected (rMVA-tmIgE/cells) tumor cells. The 100% release positive control consists of soluble anti-NIP IgE-sensitized RBL-SX38 cells cross-linked with NIP-BSA (IgE + NIP-BSA). NC, nonstimulated RBL-SX38 cells; IgE, human soluble anti-NIP IgE-loaded RBL-SX38 cells in the absence of NIP-BSA. Values are mean $\pm$ SD of three determinations.

Simultaneous determination of in vivo tmIgE antitumor adjuvant activity and confirmation of Fc ϵ RI key role in this process

After their in vitro quality control, the rMVA-tmIgE-infected TS/A-LACK cells were used to vaccinate Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ mice (14). In these transgenic mice, the Fc ϵ RI α -chain gene has been

substituted with the human counterpart by the intermediate generation of a knockout, the Fc ϵ RI $^{-/-}$ mouse (15), a crucial strain in the demonstration of the role played by Fc ϵ RI in IgE adjuvanticity (10). Therefore, Fc ϵ RI $^{-/-}$ mice were used in this study as the most appropriate control for the Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ mice. Mice were vaccinated with MVA-Green- or rMVA-tmIgE-infected TS/A-LACK cells and challenged 15 d after the immunization with living TS/A-LACK tumor cells. Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ mice immunized with the rMVA-tmIgE/TS/A-LACK cellular vaccine showed a significant antitumor effect in tumor growth ($p < 0.0001$, as compared with mice immunized with the control vaccine, not expressing tmIgE) (Fig. 4A). Regarding survival (Fig. 4B), comparing the control vaccine group with the nonimmunized mice, no significant difference was observed ($p = 0.825$), whereas comparing the rMVA-tmIgE/TS/A-LACK cellular vaccine with the control vaccine, a borderline p value of 0.0648 was obtained. However, a significant p value (0.0391) was obtained when comparing the tmIgE-based vaccine with the pool of control vaccine and nonimmunized mice. These results demonstrate that IgE can exert its antitumor adjuvanticity also in the tmIgE format, the core of this work. A second fundamental aspect is represented by the need to confirm the key role of Fc ϵ RI. Therefore, Fc ϵ RI $^{-/-}$ mice were used as control strain and treated following the same protocol used for the Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ strain (Fig. 4C). As expected, the antitumor protection exerted by the cellular vaccine in Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ mice was completely lost in Fc ϵ RI $^{-/-}$ mice (there was no significant difference in tumor growth in nonimmunized animals and in animals immunized with MVA-Green or rMVA-tmIgE). Indeed, lack of a tmIgE effect on Fc ϵ RI $^{-/-}$ mice provides a further confirmation for the crucial role played by Fc ϵ RI in IgE antitumor adjuvanticity. However, given the absence of binding by human IgE toward mouse Fc ϵ RI (1, 10), wild-type BALB/c mice would have been sufficient to provide an NC for these in vivo experiments.

Human secretory and membrane IgE (in the tmIgE format) have comparable antitumor adjuvant activity

Following the demonstration of the rMVA-tmIgE antitumor adjuvanticity and the tmIgE-Fc ϵ RI interaction as the molecular trigger of this effect, the tumor protection induced by this engineered cellular vaccine was compared with the reported cellular vaccine based on IgE-loaded MVA-Green-infected TS/A-LACK cells (10). In other words, the antitumor potency of human IgE was tested both in its secretory and membrane form. Fc ϵ RI $^{-/-}$ hFc ϵ RI $^{+}$ mice were vaccinated with rMVA-tmIgE-infected TS/A-LACK cells or IgE-loaded MVA-Green-infected TS/A-LACK cells and challenged 15 d after the immunization with living TS/A-LACK tumor cells. As for the experiments reported in Fig. 4, MVA-Green-infected tumor cells were used as control. Mice immunized with the tmIgE-based and IgE-loaded cellular vaccines showed tumor protection when compared with the mice immunized with the control vaccine ($p = 0.0188$ for the tmIgE and $p = 0.0015$ for the soluble IgE group). Remarkably, the two mice groups showed very similar tumor growth ($p = 0.2941$), demonstrating that tmIgE is able to induce an antitumor adjuvant effect of the same potency of that produced by soluble IgE (10) (Fig. 5A). Mice survival (data not shown) in the tmIgE-based and IgE-loaded cellular vaccine groups was also comparable ($p = 0.8004$) and different (extended survival) from the survival of the control group ($p = 0.0045$ for tmIgE). Moreover, wild-type BALB/c mice, used as control, were vaccinated with MVA-Green- or rMVA-tmIgE-infected TS/A-LACK cells and challenged 15 d after the immunization with living TS/A-LACK tumor cells, showing no protection and no differences in tumor growth (Fig. 5B) and sur-

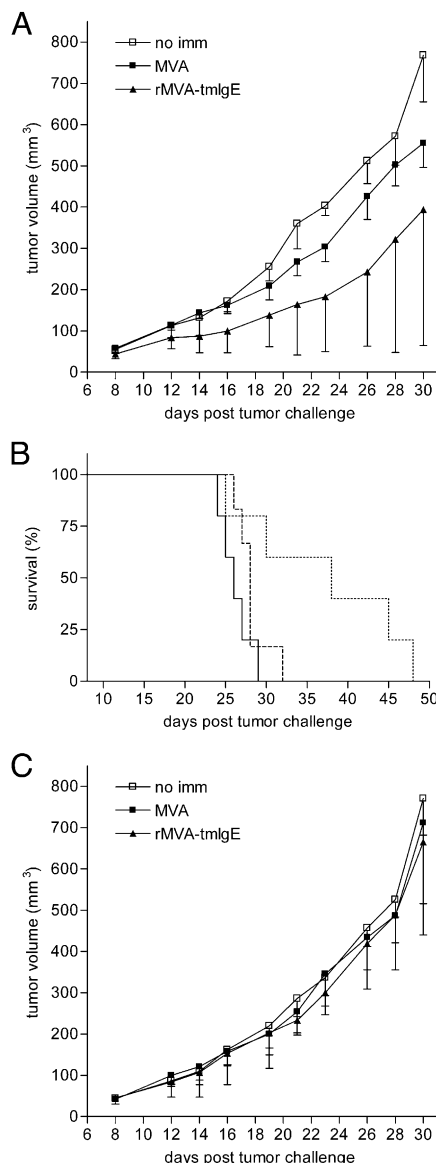


FIGURE 4. Antitumor adjuvant effect of tmIgE via the tmIgE-FcεRI interaction. **A**, FcεRI $\alpha^{-/-}$ hFcεRI α^{+} mice were vaccinated s.c. with 10^5 MVA-Green-infected (MVA) or rMVA-tmIgE-infected (rMVA-tmIgE) TS/A-LACK cells at day -15 or were not vaccinated (no imm). At day 0, all mice groups were challenged with s.c. administration of 2×10^5 living TS/A-LACK cells. **B**, Survival curve of FcεRI $\alpha^{-/-}$ hFcεRI α^{+} mice related to the experiment in **A**. Continuous line, mice not vaccinated; dashed line, mice vaccinated with MVA-Green-infected TS/A-LACK cells; and dotted line, mice vaccinated with rMVA-tmIgE-infected TS/A-LACK cells. Mice were considered dead when tumor reached 500 mm³ in volume. **C**, FcεRI $\alpha^{-/-}$ mice were treated using the same protocol as in **A**. Results are mean $\pm$ SEM and obtained from one of three experiments yielding similar results.

vival (data not shown). Again, these results attest that tmIgE present an antitumor effect based on the tmIgE-FcεRI interaction and that this effect is comparable to that obtained using secretory IgE, strengthening the value of a compact rMVA-tmIgE antitumor vaccine vector.

Discussion

Over the last 20 y, several studies reported IgE-related antitumor effects (6). It would therefore be very interesting and exciting to isolate endogenous IgE with TAA specificity; initial efforts in this direction are being made (8). Thus, studying endogenous IgE as

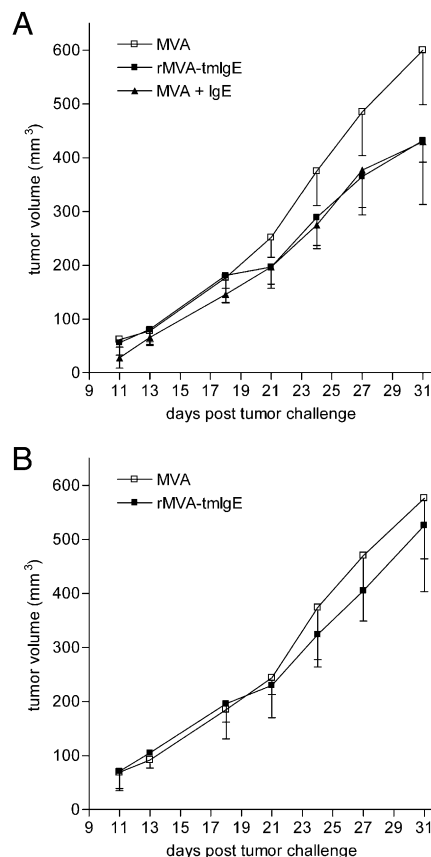


FIGURE 5. Comparable antitumor adjuvant effect induced by rMVA-tmIgE- and IgE-loaded MVA-Green-infected TS/A-LACK cells. **A**, FcεRI $\alpha^{-/-}$ hFcεRI α^{+} mice were vaccinated s.c. with 10^5 MVA-Green-infected (MVA), rMVA-tmIgE-infected (rMVA-tmIgE), or IgE-loaded and MVA-Green-infected (MVA + IgE) TS/A-LACK cells at day -15. At day 0, all mice were challenged with s.c. administration of 2×10^5 living TS/A-LACK cells. **B**, As a control of the experiment in **A**, BALB/c wild-type mice were vaccinated s.c. with 10^5 MVA-Green-infected or rMVA-tmIgE-infected TS/A-LACK cells at day -15. At day 0, all mice were challenged with s.c. administration of 2×10^5 living TS/A-LACK cells. Results are mean $\pm$ SEM and obtained from one of three experiments yielding similar results.

a tumor surveillant represents a very stimulating avenue. Following this line of thought, IgE has been exploited as antitumor agent in several experimental therapeutic protocols (6, 7).

Aside from its direct antitumor potency, or, even better, in addition to that, IgE has been investigated for its adjuvant effect. Research on IgE as antitumor adjuvant witnessed a stepwise evolution, both in terms of tumor cell-targeting protocols, tumor cell lines, mouse strains, and molecular formats (6, 7, 9, 10). Encouraging preliminary experiments in which VV was used instead of cell irradiation, to assure vaccinating tumor cell death (7), led to the use of MVA, a highly attenuated form of VV with a severe host restriction (31). Lack of pathogenicity for mammals, high-level expression of foreign Ags, its adjuvant effect on immune responses, and the possibility to produce recombinants with ease (12, 13) make MVA an ideal vector for both prophylactic and therapeutic vaccination (32). Hence, MVA has been coupled to mouse and human IgE in antitumor cellular vaccines (6, 7, 10). In this report, human IgE has been used in a novel format: its membrane-bound form has been engineered to obtain a mini-membrane IgE (11). Interestingly, this is an unusual case of Ab engineering, as it witnesses the rarely used IgE isotype and a conceptually different single-chain Ab format, not based on the

Ag binding site but on the FcR binding site (Fig. 1). This innovative concept stems from the cellular and biochemical demonstration of a functional interaction between mIgE and FcεRI (11). Indeed, the cell membrane environment provides the necessary local concentration for mIgE to be able to aggregate and activate FcεRI on the surface of a different cell in a cell-to-cell dialogue, even in absence of the Ag (11). Although this feature needs to be better characterized and understood for its biological and immunological significance, it provided the proof of principle for its biotechnological use in an antitumor cellular vaccine. Therefore, an rMVA was produced that encoded for tmIgE, a truncated single-chain Fc (scFc) mIgE. The scFc is different from the scFv for two main features: 1) it is composed of a naturally occurring Ab Fc portion instead of the classically engineered Fv moiety; and 2) its single-chain status is temporary because, once expressed, it self-assembles into dimers. In addition to the above, tmIgE is a membrane-bound scFc, in which the membrane anchorage is a fundamental requisite for its use in this report. Therefore, whereas an scFc format could be proposed for any Ab isotype (e.g., as FcR binding Ab inhibitors/competitors), the membrane-bound scFc version used in this study is to date exclusively reserved for human IgE. This is due to the fact that human mIgE does not necessarily need the Igα and Igβ accessory proteins to be transported to the cell surface, and mIg-FcR binding has been reported only for IgE (11). Yet, mIgM binding through its Fc portion to C1q has been revealed (33), strengthening the hypothesis that a functionally competent Fc binding region might be present in all mIgs (11). Going back to the biomedical use of this IgE feature, the possibility of neglecting Igα-Igβ heterodimers for mIgE (and thus tmIgE) surface expression is fundamental for its use as antitumor adjuvant, as it allows its expression on any cell type.

The production of rMVA-tmIgE in CEF, with tmIgE characterization and recombinant virus clone selection, allowed its use in TS/A-LACK cells, the BALB/c tumor line used in this report. The successful folding and FcεRI binding activity by tmIgE, already reported for a stable sp2/0 cell transfectant (11), has been confirmed also in the context of CEF infection by rMVA-tmIgE. Upon in vitro characterization and quality control of the rMVA-tmIgE/TS/A-LACK cells, these have been used to vaccinate the FcεRI-humanized FcεRIα^{-/-} hFcεRIα⁺ mice, a convenient strain to study human IgE-FcεRI interactions in vivo (10, 14). FcεRIα^{-/-} hFcεRIα⁺ mice have been characterized for their ubiquitous expression of FcεRIα (14, 34, 35), a feature that may be revealed to be precious for the similarity with the human cellular distribution of the receptor. However, awaiting a detailed cellular characterization of this IgE-based antitumor system, mast cells and basophils were reported in Fig. 1 as the main cell players, as this involvement stems from the mouse IgE system evidence (6, 7, 9, 10). FcεRIα^{-/-} as well as wild-type BALB/c mice have been used as controls of the cellular vaccine efficacy. The in vivo experiments led to the following evidence: 1) the proof of tmIgE antitumor adjuvanticity, provided by the reduction of tumor growth rate, as compared with controls; 2) the dependence of these effects on the tmIgE-FcεRI interaction, provided that only the FcεRI-humanized mouse allows human IgE to interact with FcεRI; and 3) an antitumor potency exerted by tmIgE comparable to that of soluble IgE. Overall, the advantages gained upon a shift from soluble IgE to tmIgE in a rMVA-encoding vector open the possibility for further developments in the direction of IgE adjuvanticity. Clearly, the use of tmIgE eliminates any unwanted or unpredictable anaphylactogenicity by the IgE molecule, because the membrane anchorage would prevent soluble IgE release and circulation in the bloodstream. As a matter of fact, no allergic-like

symptoms (dermatological lesions or scratching behavior) have been observed to date in mice receiving either soluble IgE or tmIgE. In addition to the above, the lack of Ag binding sites on tmIgE eliminates de facto any possible Ag binding as well as cross reactivity. Ultimately, the rMVA-tmIgE represents a compact and safe antitumor vaccine vector lead.

Coupling safety and wide use of MVA as vaccine vectors, fast rMVA production technology, and tmIgE efficacy as antitumor adjuvant allows us to reason upon the possibility to coexpress tmIgE with a second antitumor protein (e.g., a T cell stimulatory factor) in an rMVA context. In conclusion, this work reports the transduction of the biochemical and cell biological evidence for the mIgE-FcεRI interaction into a biotechnological tool for antitumor adjuvanticity via the rMVA technology.

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Disclosures

The authors have no financial conflicts of interest.

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