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J Immunol published online 26 August 2016

<http://www.jimmunol.org/content/early/2016/08/26/jimmunol.1601026>

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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'-GAAATG-GACCTATAAGCTTAGAGCCTTCC-3', h reverse, 5'-GATGTTCTTC-

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Received for publication June 14, 2016. Accepted for publication August 11, 2016.

V.A.Y. was supported by a Ph.D. fellowship from the Graduate School in Pharmacological Sciences, Università degli Studi di Milano. The European Federation of Immunological Societies awarded a short-term fellowship to E.A.N., and the Fondazione Anna Villa e Felice Rusconi provided support to V.A.Y. and L.M.F. Also, the Armenise-Harvard Foundation provided support to E.v.A.

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

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TAAGCTTTGTCTCAAAG-3'; H forward, 5'-GAATGGGCTGACCGC-TTCCTC-3', H reverse, 5'-GGTAAAGGCTGCTTCCTAGTC-3'; R forward, 5'-TTGGCTGCTCCTTCAGACAT-3', R reverse, 5'-CTCTC-TAATGGAGACGGGGC-3'; r forward, 5'-CTTGGGTGGAGAGGCTA-TTC-3', r reverse, 5'-CTCTCTAATGGAGACGGGGC-3'. The PCR produced DNA amplicons of 930, 1200, 161, and 1300 bp for h, H, R, and r, respectively.

Cell lines

TS/A is a mammary adenocarcinoma cell line of BALB/c origin (16). N2C is a primary mammary carcinoma cell line derived from BALB-neuT mice (17). RBL-2H3 is a rat basophilic leukemia cell line.

Tumor injection and monitoring

Immunizations were performed on five mice for each group by s.c. administration of irradiated (10,000 rad) TS/A tumor cells (10^5 cells/mouse) in PBS at the tail base. Two weeks after immunization, mice were s.c. challenged with 2×10^5 TS/A cells on the abdomen. In the N2C model, mice were challenged s.c. with 6×10^5 tumor cells. Tumor growth was monitored at 1- to 3-d intervals by measuring tumor volume with a caliper as already described (11, 12). Animals were euthanized when the tumors became >500 mm³.

Histology

At different time points, tissues where tumor cells had been injected were collected, fixed in buffered 4% formalin, and embedded in paraffin. Multiple 3- μ m sections from paraffin-embedded samples were cut and stained with H&E for microscopic analysis. Photomicrographs were taken using the AxioCam HRc with the AxioVision Vs40 v.4.6.3.0 (Zeiss).

Mouse serum collection and processing

Serum was obtained from peripheral blood collected by tail vein puncture. To deplete IgE, an ELISA plate was coated with 10 μ g/ml rat anti-mouse IgE (clone R35-72; BD Biosciences). One hundred microliters of serum was then incubated three times on the anti-IgE-coated ELISA wells for 30 min at room temperature. To allow tumor-specific IgE binding to tumor cells, 2×10^5 N2C cells in 100 μ l of DMEM were incubated with 50 μ l of serum for 1 h at 4°C and then washed three times with PBS.

Mediator release assay

RBL-2H3 cells (8×10^4 cells/well) were incubated in DMEM with 100 ng of mouse IgE κ isotype control Ab (clone C38-2; BD Biosciences) for 2 h at 37°C. Cells were then washed and incubated in triggering buffer (119 mM NaCl, 5 mM KCl, 26 mM PIPES, 5.6 mM glucose, 1 mM CaCl₂, 0.4 mM MgCl₂, 0.1% BSA [pH 7.2]) with 100 ng of anti-mouse IgE mAb (R35-72) for 1 h at 37°C (positive control). Alternatively, 2×10^5 serum-preincubated N2C tumor cells were washed, resuspended in triggering buffer, and added to RBL-2H3 cells. Plates were centrifuged 5 min at $300 \times g$ and incubated for 1 h at 37°C. The release of β -hexosaminidase was monitored on RBL-2H3 cell supernatant incubated with 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide (Sigma-Aldrich) in 0.1 M citrate buffer (pH 6.2) for 150 min at 37°C. The reaction was stopped by adding 0.1 M stop solution (0.1 M Na₂CO₃, 0.1 M NaHCO₃ [pH 10]), and the absorbance was read at 405 nm (18). Results are calculated as percentage of the positive control (IgE plus crosslinker).

In vivo CD8⁺ T cell depletion

KN1 mice were depleted of CD8⁺ T cells by i.v. injections of 300 μ g of rat anti-mouse CD8 α ascite (clone 53-6.7; Abnova) at days -6, -3, and -1 before tumor challenge. CD8⁺ T cell depletion was monitored by cytofluorimetric analysis on peripheral blood.

Statistical analysis

Tumor volume growth curves were fitted by means of nonlinear mixed effects models. To account for the dependence among longitudinal observations as well as for possible undetectable initial differences, tumor growth curves were estimated by an exponential mixed effect model with multiplicative errors: $V(t) = \alpha_0 e^{\alpha_i t} \varepsilon$, where V is the volume of the tumor at time t , ε has log-normal distribution, and i is the experimental condition (11). The model was fitted by restricted maximum likelihood (19). The model allows an easy interpretation of tumor growth results because α_0 is related to the volume of the initial tumor volume and α_i is related to their growth speed. Furthermore, multiplicative log-normal distributed errors address for the higher variability observed in correspondence of higher mean values.

Survival curves were fitted by means of the Kaplan–Meier estimator, and differences between survival curves were evaluated by means of a log-rank test.

The obtained p values were adjusted for false discovery rate with the Benjamini and Hochberg (20) method.

For β -hexosaminidase activity, the differences between absorbance levels were assessed by means of the Welch statistic, and p values were also adjusted for false discovery rate. Data analysis and graphic representation were developed in the R environment (version 3.1.2). Figures were prepared using Prism 5.04 (GraphPad Software).

Results and Discussion

IgE-dependent susceptibility to tumor growth

To explore a possible tumor immunosurveillance role by endogenous IgE, we employed a mouse model (IgE-KO) in which part of the IgE H chain locus (comprising the ε -M1-M2 exons) is deleted, preventing the switch to the IgE isotype without evident effects on general immunocompetence (15). The IgE-KO was produced in a BALB/c background for which TS/A, a mammary adenocarcinoma cell line of BALB/c origin, is a well-established tumor model (16). We challenged both IgE-KO and matching wild-type (WT) mice by s.c. injection of TS/A tumor cells. After tumor cell engraftment, tumor volume progression in WT mice was comparable to that reported in a previous study (11) and identical to that observed in IgE-KO mice (Fig. 1A). However, when we immunized mice with irradiated TS/A cells 15 d prior to the challenge with live tumor cells, WT mice tumor volumes were significantly smaller ($p = 0.012$) (Fig. 1A) and their survival was prolonged by 10 d (Fig. 1B). Conversely, in the immunized IgE-KO mice, tumor growth (Fig. 1A) and survival (Fig. 1B) were unaffected, supporting a scenario in which protection in WT mice was provided by an antitumor IgE response raised through the immunization with irradiated TS/A cells.

Having established that the lack of IgE response enhanced tumor growth in immunized mice, we next asked whether reinforcement of the IgE response could also affect tumor growth. To this end, we exploited the BALB/c KN1 mouse model (13), in which the ε -M1-M2 exons are replaced by γ 1-M1-M2 exons, resulting in the expression of chimeric IgE-IgG1 at the surface of B cells (see insert in Fig. 1A). Notably, the secreted Ab is a genuine IgE, but the γ 1-anchored B cell receptor causes an alteration in homing and survival of plasma cells, with respect to the ε -anchored receptor. Therefore, the KN1 mouse model is particularly suitable for this study because the number of IgE-secreting B cells is increased (up to 10-fold in the bone marrow) and serum IgE titers are 4- to 6-fold higher than in WT mice after Ag challenge (13). Upon TS/A challenge, tumor growth was remarkably reduced in KN1 mice compared with WT ($p < 0.00001$), and totally impaired when KN1 mice were first immunized with irradiated TS/A cells ($p < 0.00001$ versus immunized WT) (Fig. 1A). In agreement with the reduced tumor growth, survival of the TS/A-challenged nonimmunized KN1 mice was prolonged as compared with WT ($p = 0.043$), whereas 100% survival was observed in immunized KN1 mice ($p = 0.013$ versus immunized WT and $p = 0.041$ versus nonimmunized KN1) (Fig. 1B). Furthermore, none of the KN1 mice that survived the challenge with TS/A cells developed tumors when rechallenged (data not shown), indicating a long-term persistence of antitumor immunity.

We then extended our investigation to N2C, a primary mammary carcinoma cell line derived from BALB/NeuT mice (17), which causes death at a slower rate than does TS/A (compare WT curves in Fig. 1B and 1D). Even without

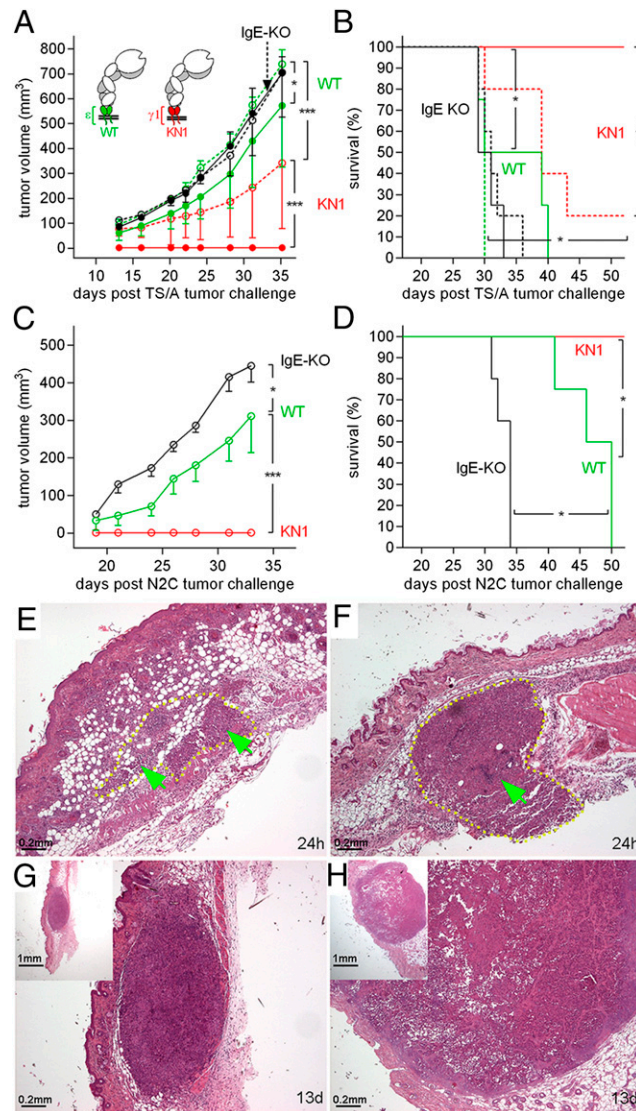


FIGURE 1. Mice lacking IgE or having strengthened IgE response show different susceptibility to transplantable tumors. (A) The insert depicts membrane IgE domain structure of WT and KN1 mice, showing the extent and position of the IgG1 fusion. Tumor growth curves: IgE-KO (black lines), WT (green lines), and KN1 (red lines) mice were challenged with living TS/A cells without (○, dashed lines) or with (●, solid lines) prior immunization with irradiated TS/A cells. (B) Survival of IgE-KO, WT, and KN1 mice (nonimmunized, dashed lines; immunized, solid lines) as in (A). (C) Tumor growth curves: IgE-KO (black line), WT (green line), and KN1 (red line) mice were challenged with living N2C cells without prior immunization. (D) Survival of the same mice as in (C). Statistical analysis: tumor volume growth curves were estimated by a nonlinear mixed effect model and with survival by Kaplan–Meier analysis (log-rank test). Shown are representative experiments out of three to four replicates. In (A) and (D), $n = 5$ mice. Error bars indicate SD. (E–H) H&E-stained sections of N2C tumors at 24 h after injection in KN1 (E) and WT mice (F) and at 13 d after injection in KN1 (G) and WT mice (H). Tumor areas in (E) and (F) are encircled by small yellow squares and indicated by arrows. * $p < 0.05$, *** $p < 0.001$.

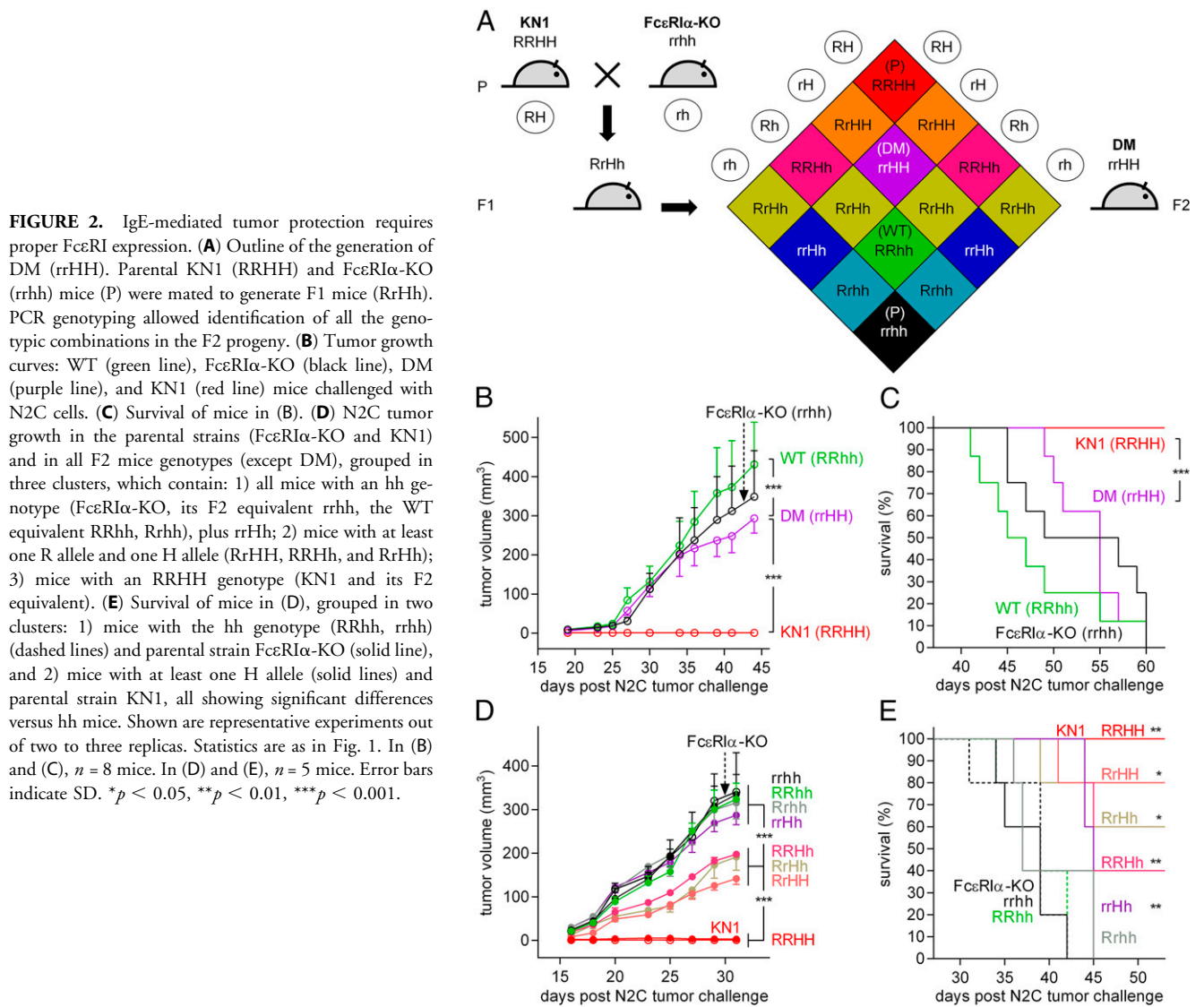
prior immunization, N2C tumor growth was significantly higher in IgE-KO than in WT ($p = 0.012$), whereas KN1 mice showed no tumor growth at all ($p < 0.00001$ versus WT) (Fig. 1C). Accordingly, IgE-KO mice died 15 d earlier than did WT ($p = 0.023$), whereas KN1 mice showed 100% survival ($p = 0.043$ versus WT) (Fig. 1D). Histological analysis 24 h after N2C cell challenge revealed tumor cell

engraftment in KN1 and WT mice (Fig. 1E and 1F, respectively), indicating proper tumor take. After 13 d, when the tumors became palpable, only small tumors were detected in KN1 mice (Fig. 1G), whereas tumors in WT mice were larger (Fig. 1H). In the following days, tumors in KN1 mice completely regressed, whereas tumors in WT mice grew steadily. Thus, the analysis of two independent tumor models indicates the existence of an IgE-driven tumor immunosurveillance.

Predominance of the IgE–FcεRI axis in the IgE-mediated tumor protection

In mice, IgE exerts its effect in at least two directions, one offered by high-affinity interaction with FcεRI (5) on the surface of mast cells and basophils, and the other by low-affinity interaction with CD23 on B cells and APCs (21). We previously established that the adjuvant effect of IgE in tumor vaccination is dependent on FcεRI and not on CD23 (11). Thus, we tested whether also endogenous IgE relies on the IgE–FcεRI axis to exert antitumor effects. For this purpose, we removed the FcεRI in KN1 mice by a cross with FcεRIα-KO mice (14) to generate DM mice (KN1/FcεRIα-KO). For simplicity, we named alleles for normal FcεRIα and for FcεRIα-KO R and r, and alleles for normal and strengthened IgE response h and H, respectively. PCR genotyping allowed identification of all the expected genotypic combinations in the F2 progeny, including the DM rrHH (Fig. 2A). As shown in Fig. 2B with N2C challenge, the protective effect linked to the KN1 genotype (RRHH) was largely lost in DM mice ($p < 0.0001$ versus KN1 and F2-equivalent mice). Thus, the IgE–FcεRI axis appears as the main mechanism of action of IgE. The original parental strains FcεRIα-KO and KN1 and their genotypic equivalent F2 mice (rrhh and RRHH, respectively) displayed identical tumor growth (Fig. 2D) and survival (Fig. 2E), indicating that no unidentified loci are involved in the generation of the tumor-protected phenotype of KN1 mice. As expected, also the WT and F2-equivalent RRhh mice presented identical tumor growth and survival (data not shown).

Interestingly, mice retaining at least one H allele and one R allele (i.e., RRHh, RrHh, and RrHH) displayed intermediate protection in N2C tumor growth ($p < 0.00001$ versus the WT-equivalent RRhh, FcεRIα-KO and its F2 equivalent rrhh) (Fig. 2D). This finding was mirrored in the survival curves, as F2 mice with at least one H allele and one R allele showed better survival than did the WT-equivalent RRhh ($p = 0.03$, $p = 0.035$, and $p = 0.008$, respectively) (Fig. 2E). In particular, longer term survival strictly depended on the presence of at least one H allele: HH homozygotes RRHH and RrHH yielded five of five and four of five survivors, respectively. Hh heterozygotes RrHh and RRHh yielded three of five and two of five survivors. At least one R allele is necessary for H-dependent longer term survival, because both the DM rrHH (Fig. 2C) and rrHh (Fig. 2E) yielded no survivors of five. We conclude that high-affinity interactions with FcεRI provide the major axis through which IgE exerts tumor immunosurveillance. Of note, tampering with the H alleles offers an example of genetic titration: maximal immunosurveillance was observed only with a full complement of R and H alleles, as supported by the differences ($p < 0.00001$) in tumor growth rate of RRHH versus RRHh, RrHH, and RrHh (Fig. 2D).



In agreement with the predominance of the IgE–FcεRI axis, a previous study showed that CD23-KO mice (22) have no increase in tumor susceptibility compared with WT (11). Still, in the absence of FcεRI, IgE could be rerouted toward alternative protective mechanisms. In line with this reasoning, H alleles conferred some reduction of tumor growth also in DM (rrHH) mice in which FcεRI is absent ($p = 0.0003$ compared with WT) (Fig. 2B). Thus, alternative IgE-mediated mechanisms could explain why FcεRIα-KO and IgE-KO mice do not respond in the same way to the N2C tumor challenge, as it would be expected if IgE–FcεRI were the only axis through which IgE could exert its antitumor effect. Therefore, a concomitant minor role played by CD23 might not be excluded.

Detection of a tumor-reactive IgE response

Our findings that tumor immunosurveillance in IgE-KO mice is decreased and remarkably enhanced in KN1 mice strongly suggest that mice raise antitumor IgE when challenged with tumor cells. Although we failed to isolate tumor-specific hybridomas from either spleen or bone marrow of KN1 mice that rejected the tumors, we produced evidence of the existence of

tumor-reactive IgE by a functional assay we previously developed (18). N2C cells were incubated with sera from tumor-challenged WT, FcεRIα-KO, KN1, and DM mice and mixed with RBL-2H3 rat basophilic leukemia cells. Tumor-reactive IgEs are expected to bind to N2C surface Ags, which in turn aggregate FcεRI on the surface of RBL-2H3 cells, resulting in the release of mediators of the allergic response, including β-hexosaminidase. Preincubation of N2C cells with IgE/κ isotype control Ab did not yield a significant β-hexosaminidase release, whereas cross-linked IgE led to a massive release (Fig. 3). Sera from tumor-challenged WT and FcεRIα-KO failed to induce mediator release over background levels; in similar experiments, no difference between WT and IgE-KO sera could be detected (data not shown). Conversely, sera from tumor-challenged KN1 and DM mice triggered β-hexosaminidase release significantly higher than background ($p = 0.003$). Depletion of IgE from these sera completely abolished mediator release ($p = 0.004$ for KN1 and $p = 0.02$ for DM), validating the contribution of IgE (Fig. 3). Equivalent release values were obtained after a second (Fig. 3) tumor challenge to KN1 mice, demonstrating the persistence of the tumor-reactive IgE response.

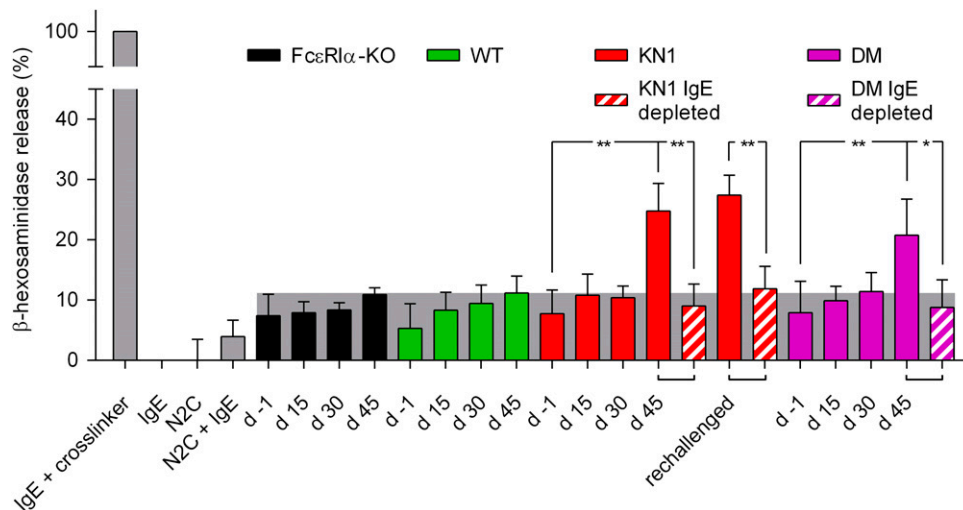


FIGURE 3. Tumor growth elicits tumor-reactive IgE. RBL-2H3 cellular release of β -hexosaminidase was monitored after mixing and incubating with N2C cells treated with sera from KN1, rrHH, Fc ϵ RI α -KO, and WT mice sampled before and after N2C cell challenge. The average release value of sera collected 1 d before tumor challenge ($n = 16$; mean = 7.09; SD = 4.20) was set as background (mean + SD = 11.29) (gray bar). Shown is a representative experiment out of three replicas. Statistical significance of the positive values (by sera of KN1 and DM mice at 45 d after challenge, and for KN1 30 d after a second tumor challenge) was determined by a Welch t test in comparison with background, or with IgE-depleted samples (striped columns); $n = 3$ mice. Error bars indicate SD. * $p < 0.05$, ** $p < 0.01$.

Depletion of CD8⁺ T cells impairs IgE-dependent tumor protection

We had previously shown that the adjuvant effect of exogenous IgE on tumor vaccination depends on CD8⁺ T cells (10). Therefore, we depleted CD8⁺ cells in KN1 mice by three sequential injections of anti-CD8 Abs before challenging the KN1 mice with the N2C tumor cells. Depletion (>80%) of CD8⁺ cells allowed N2C tumor growth in KN1 mice ($p < 0.00001$) (Fig. 4). We conclude that IgE tumor surveillance is largely mediated by a CD8-dependent immune response.

In conclusion, the present study shows that endogenous IgE contributes to tumor immunosurveillance by the IgE–Fc ϵ RI axis and that CD8⁺ T cells are crucial for the tumor-protective effect. Although the mechanism that links IgE and CD8⁺ T cell activation remains to be defined, a recent report shows that IgE-driven Ag cross-presentation by mouse dendritic cells engineered to express human Fc ϵ RI induces the generation of antitumor cytotoxic CD8⁺ T lymphocytes (23). As Fc ϵ RI is not expressed on murine dendritic cells, we speculate that other Fc ϵ RI⁺ cells must be involved. Murine mast cells could

promote T cell activation via the release of exosomal material, including IgE/Ag complexes (24, 25). It is tempting to speculate that IgE-mediated tumor surveillance might be even more effective in humans, as human dendritic cells express Fc ϵ RI and could participate directly in the response.

IgE is highly conserved in the mammalian lineage, likely for its role in the defense against parasites. The strength of the IgE response may therefore be an evolutionary compromise, fine-tuned to convey antiparasitic and tumor surveillance activity. Slight deviations from the optimal IgE response may explain the high incidence of atopy, which, interestingly, does seem to correlate with a lowered risk for developing cancer (9, 26, 27).

Because of the prevalence of atopy-related pathologies, most research efforts are directed at dampening the IgE response but, in light of our data, enhancing tumor-specific IgE responses may offer an interesting avenue toward antitumor therapies (28).

Acknowledgments

We thank Anna Mondino, Mario Colombo, and David Dombrowicz for reagents and mouse strains and Martina Rocchi and Anna Innocenzi for technical assistance. We dedicate this work to the memory of Gernot Achatz, who is sorely missed.

Disclosures

The authors have no financial conflicts of interest.

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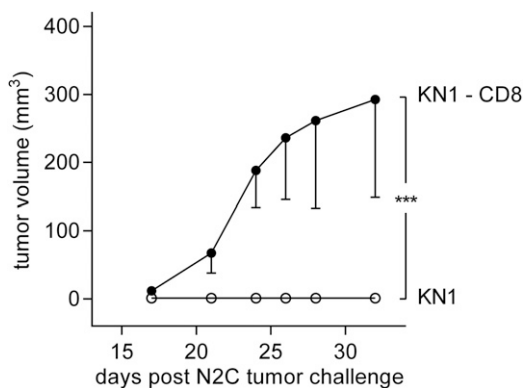


FIGURE 4. IgE-dependent protective immunity requires CD8⁺ T cells. N2C tumor growth curves of untreated (○) and CD8-depleted (●) KN1 mice. Shown is a representative experiment out of two replicas. Statistics are as in Fig. 1; $n = 5$ mice. Error bars indicate SD. *** $p < 0.001$.

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