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Anti-tumor immunization of mothers delays tumor development in cancer prone offspring

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List of abbreviation: Amot, Angiomotin p80; BALB-neuT mice, BALB/c mice heterozygous for the transforming form of the neu transgene; BKO mice, BALB/c female mice KO for the μIg chain; ECTM, extracellular and transmembrane; FcγKO mice, BALB/c mice KO for the Fc-gamma I/III receptors; FcγRI/III, Fc-gamma I/III receptors; IFN-γ, interferon- γ; KO, knock out; LNs, lymph

nodes; RSI, rate of stimulation index; SFU, spot-forming unit; SPC, splenocytes; Treg, T regulatory cell.

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Abstract

Maternal immunization is successfully applied against some life-threatening infectious diseases as it can protect the mother and her offspring through the passive transfer of maternal antibodies. Here we sought to evaluate whether the concept of maternal immunization could also be applied to cancer immunoprevention. We have previously shown that antibodies induced by DNA vaccination against rat Her2 (neu) protect heterozygous neu-transgenic female (BALB-neuT) mice from autochthonous mammary tumor development. We herein seek to evaluate whether a similar, maternal, immunization can confer anti-tumor protection to BALB-neuT offspring. Significantly extended tumor-free survival was observed in BALB-neuT offspring born and fed by mothers vaccinated against neu, as compared to controls. Maternally derived anti-neu IgG were successfully transferred from mothers to newborns and were responsible for the protective effect. Vaccinated mother offspring also developed active immunity against neu as revealed by the presence of T-cell-mediated cytotoxicity against the neu immunodominant peptide. This active response was due to the milk transfer of immune-complexes that were formed between the neu extracellular domain, shed from vaccine-transfected muscle cells, and the anti-neu IgG induced by the vaccine. These findings show that maternal immunization has the potential to hamper mammary cancer in genetically predestinated offspring and to develop into applications against lethal neonatal cancer diseases for which therapeutic options are currently unavailable.

Introduction

Vaccination is the most powerful and versatile tool in preventive medicine and has recently extended its reach beyond infectious diseases to other life-threatening illnesses, such as cancer. Examples can be found in vaccines which have been designed to prevent infection-associated tumors [1] and in those targeting non-infection-related cancer diseases now in pre-clinical phases or in clinical trials [2].

Maternal immunization against life-threatening disease inducing pathogens has been shown to be a viable approach against many childhood pathologies. The induction of high antibody levels following vaccination is fundamental to maternal immunization success. It has long been known that maternally derived IgG is the only antibody class that causes short-term passive immunity to be transferred from mother to fetus across the placenta and via the proximal small intestine during breastfeeding [3, 4]. This passive protection slowly declines over the first year of life while the infant's immune system become more mature [5].

The vaccination of pregnant women against tetanus and the influenza virus has recently been proven to be safe and highly effective in providing newborn children with maternally transferred antibodies and thus protection from pathogens [6-8]. Several antenatal vaccines are now available and recommended for pregnant women while others are in development [9, 10]. Maternal immunization against tumor-associated antigens instead is not a currently rated field and very few pre-clinical attempts have been made to prevent neonatal congenital tumors by maternal vaccination [11, 12].

Most of our recent studies have focused on DNA vaccination against what we have defined as oncoantigens; tumor-associated antigens that have a causal role in the promotion of tumor progression [13, 14]. Membrane tyrosine kinase Her2, of the epidermal growth factor receptor family, is expressed by many human carcinomas in association with poor prognosis [15] and fulfills the definition of oncoantigen. We have demonstrated that vaccination with a plasmid that codes for the

extracellular and transmembrane (ECTM) domains of rat Her2 (neu) effectively inhibits mammary carcinogenesis [16-18] in female BALB/c mice heterozygous for the transforming form of the *neu* transgene under the transcriptional control of the mouse mammary tumor virus promoter (BALB-neuT mice) [19, 20]. Vaccine-elicited tumor inhibition in these mice is driven by anti-neu antibody generation [16, 17], while the T cell cytotoxic response is marginal as T cells that react against neu with high affinity are wiped out by central tolerance [21, 22].

The induction of high levels of antibodies following vaccination is also the mainstay of the success of maternal immunization strategy against infectious diseases. In the present study, we therefore seek to evaluate whether maternal immunization can also induce an anti-neu immune response able to hamper spontaneous tumor progression in BALB-neuT offspring.

Results

Vaccine-induced anti-tumor antibodies are transferred from mothers to their offspring and delay tumor development

Virgin BALB/c female mice were twice vaccinated via electroporation of ECTM plasmid (ECTM mothers) or its empty control vector (control mothers) and mated with a BALB-neuT male soon after their last immunization. No fertility impairment, reduction in litter number, newborn size or in the percentage of BALB-neuT mice was evident in the comparison between offspring born from and fed by control (control offspring), ECTM (ECTM offspring) and untreated mothers (data not shown). The presence of anti-neu antibodies in ECTM mother sera and milk was confirmed two weeks after the last immunization and three weeks after delivery, respectively (Fig. 1A). As expected, passively transferred anti-neu antibodies were found in the sera of ECTM offspring, but not in the sera of control offspring (Fig. 1A, B). The highest anti-neu antibody amount was found at 1 week of age, probably due to colostrum ingestion, and remained high until the 5^{th} weeks. The anti-neu antibody titer dropped from week 6 probably because of offspring weaning at 4 weeks (Fig. 1B).

We have previously shown that the anti-neu antibodies induced by ECTM vaccination of BALB-neuT females halt autochthonous mammary carcinogenesis [16, 17, 23]. Having found specific anti-neu antibodies in ECTM offspring we investigated whether these antibodies were able to inhibit mammary carcinogenesis in female BALB-neuT pups (neu^+ offspring). Indeed, neu^+ ECTM offspring showed significantly extended tumor-free survival over neu^+ control offspring (Fig. 1C). At week 23 about 35% of neu^+ ECTM offspring were free from palpable lesions whereas all neu^+ control offspring displayed at least one palpable tumor. The passage of anti-tumor immunity from mother to offspring was further confirmed by the ability of non-transgenic pups (neu^- offspring) from ECTM mothers to hamper the growth of a transplantable tumor induced by a neu^+ cancer cell line challenge (TUBO cells) [24]. While 100% neu^- control offspring developed TUBO tumors, 2 of

22 neu$^-$ ECTM offspring did not develop a palpable tumor (Table 1). Moreover, the time required for the TUBO cells to give rise to 2, 4, 6 or 8 mm mean diameter tumors was significantly longer in neu$^-$ ECTM offspring than in neu$^-$ control offspring. The 10 mm mean diameter threshold (survival time) was reached in an average time of 34.8 ± 1.4 days in neu$^-$ ECTM offspring and in 26.8 ± 1.3 days in neu$^-$ control offspring (Table 1).

To determine whether this results obtained with antenatal vaccination can be generalized, BALB/c females were vaccinated against Angiomotin p80 (Amot), an oncoantigen expressed on tumor vasculature [25], using a plasmid coding for this protein (pAmot). Vaccinated females were then mated with a BALB-neuT male and neu$^+$ offspring were evaluated for mammary tumor development. We have previously shown that vaccination induced anti-Amot antibodies impair tumor vascularization [26] and significantly delay autochthonous tumor progression [27] in female BALB-neuT mice. Post-vaccination anti-Amot antibody induction was confirmed in pAmot vaccinated mother sera and milk and in the sera of their offspring (pAmot offspring) (Fig. 2A). Mammary carcinoma onset in neu$^+$ female offspring born from and fed by pAmot vaccinated mothers was significantly delayed with respect to offspring born from and fed by control vaccinated mothers (Fig. 2B).

Finally, to demonstrate that observed anti-tumor protection was due to the choice of an oncoantigen as the DNA vaccination target, we vaccinated BALB/c females with a plasmid coding for Escherichia coli β-galactosidase (LacZ plasmid), a tumor unrelated protein. Vaccinated females were soon mated with a BALB-neuT male and carcinogenesis progression was evaluated in neu$^+$ female offspring. While anti- β-galactosidase antibodies were found in the sera of both LacZ vaccinated mothers and their offspring, no statistical difference in tumor incidence was observed between neu$^+$ female offspring born from and fed by LacZ vaccinated mothers and those born from and fed by control mothers (Fig. 2C, D).

The presence of antibodies and functional FcγRI/III is required to delay mammary carcinogenesis in ECTM offspring.

To confirm the role of antibodies in mammary carcinogenesis delay, BALB/c female mice knock-out (KO) for the μIg chain (BKO mice) [28], and thus unable to produce antibodies, were electroporated with ECTM or the empty control vector. Females were mated with a BALB-neuT/BKO male a few days after the last immunization. No statistical difference in tumor incidence was observed when autochthonous mammary tumor growth was evaluated in BKO neu$^+$ ECTM and control offspring (Fig. 3A), thus proving that maternally derived antibodies are necessary for effective anti-tumor protection.

IgG2a was the most abundant IgG subclass in the milk and sera of ECTM mothers and in the sera of their pups, whereas IgG3 was the least common (Fig. 1B). This is in line with our previous findings which demonstrate that ECTM vaccination elicits the activation of T helper cells producing interferon (IFN) -γ, the primary switch factor for IgG2a [17]. IgG2a activate the complement and interact very efficiently with the Fcγ receptors on various effector cells [29].

To further elucidate how these passively transferred antibodies induce tumor delay, BALB/c mice KO for the Fc-gamma I/III receptors (FcγRI/III) (FcγKO mice) [30] were immunized with ECTM or its empty control vector and mated with a BALB-neuT/FcγKO male. FcγKO neu$^+$ ECTM offspring did not display any significant tumor onset delay over FcγKO neu$^+$ control offspring (Fig. 3B), suggesting that antibody-dependent, cell-mediated cytotoxicity is one of the mechanisms behind vaccine-induced IgG's triggering of anti-tumor protection.

neu extracellular domain (EC)-IgG immune-complexes are present in ECTM mother's milk and induce an active immune response in offspring

Transfer of antigen-IgG immune complexes with breastfeeding is an important mechanism of active immunization in the offspring. We thus set up an ELISA assay to detect EC-IgG immune-complexes, showing their presence in ECTM mothers' sera and milk (Fig. 4A). We then evaluated the presence of anti-neu IgM in the offspring sera. As shown in Fig. 4B, sera from ECTM offspring had a significant higher level of IgM over control. Moreover, exploiting a B cell ELISPOT analysis, a significantly higher amount of neu-specific IgM^+ memory B cells was found in the spleen of 5 week-old ECTM offspring as compared to age-matched control offspring (Fig. 4C).

A further confirmation of an active immune response against neu in ECTM offspring came from the evaluation of the T cell response. As expected, ECTM mothers displayed increased *in vivo* cytotoxic activity against spleen cells, pulsed with p63-71, over control mothers (Fig. 5A). In a similar assay, no specific cytotoxic response was found against p63-71 in control offspring, whereas it was evident in the ECTM offspring (Fig. 5A). Surprisingly, no significant differences in lysis percentage were found in neu^- and neu^+ ECTM offspring, while a lower, if any, cytotoxic response was expected in neu^+ ECTM offspring. We then checked the TCR repertoire used to react against p63-71 in these mice in order to shed light onto the origin of the cytotoxic activity found in the pups. Popliteal, inguinal and mesenteric LNs were collected from ECTM mothers 5 weeks post delivery and from their 5-week old offspring and the expansion of specific p63-71 TCR repertoires of $CD8^+$ T cells was evaluated. We have previously identified a public TCR rearrangement, the Vβ9-Jβ1.2 recombination, elicited by ECTM vaccination in BALB/c mice that recognizes the p63-71 peptide with high avidity and is wiped-out by central tolerance in BALB-neuT mice [21]. The expansion of this repertoire was found in all ECTM mothers as well as in 3 out of 5 neu^- ECTM offspring while, as expected, none of the neu^+ ECTM offspring presented this TCR rearrangement (Fig. 5B). We then evaluated the expansion of the Vβ6-Jβ2.7 rearrangement. This is a low avidity $CD8^+$ T cell clone that is specific for p63-71 and is induced by ECTM vaccination, but normally controlled by peripheral tolerance mechanisms such as T regulatory cell (Treg) expansion in adult

BALB-neuT mice [22]. Vβ6-Jβ2.7 rearrangement expansion was found in 5 out of 7 neu^+ ECTM offspring and in none of the neu^+ control offspring (Fig. 5B). $CD8^+$ T cell activation in ECTM offspring was also confirmed *in vitro* with an IFN-γ-based ELISPOT assay after spleen cell stimulation using the p63-71 peptide (Fig. 5C). Again, no statistically significant difference between neu^- and neu^+ ECTM offspring was found.

Discussion

The data reported in this paper show that maternal DNA immunization against two oncoantigens, neu and Amot, impairs the onset of mammary tumors in cancer-prone neu^{+} offspring. After DNA vaccination, high levels of IgG against the target oncoantigen can be detected both in vaccinated mothers' sera and milk, although at lower levels in the milk. We observed that vaccine-induced IgG were successfully transferred to the offspring, with the highest amount of specific IgG found in one week-old pup sera. A significant decrease in antibody titer was detected from the second to the 5th week of age, after which it dropped out. The passive transfer of maternal antibodies, in humans and other mammals, occurs via placental and milk transfer of IgG through the neonatal Fc receptor (FcRn) and via polymeric IgA milk transfer. In humans, FcRn is expressed by syncytiotrophoblast where it antenatally transports IgG from maternal circulation to the fetal capillaries of the placental villi [4]. By contrast, in rodents the FcRn functions most efficiently in the neonatal period when it transports maternally derived IgG in ingested milk across the epithelial-cell layer of the proximal intestine [4].

Besides being the main source of passive immunity in very early life, breastfeeding is also an important route for active immunization thanks to the efficient transfer of antigen-IgG immune complexes, contained in the milk, to the breastfed pups via the FcRn and across the proximal intestine [31]. We thus hypothesized that vaccine-transfected muscle cells might be the source of the neu protein, otherwise not present in a wild type mouse. As happens normally, EC may be shed from transfected muscle cell membranes [32], form complexes with vaccine-induced anti-neu antibodies, accumulate in the milk and be passed to the pups, triggering an active immune response. To confirm this hypothesis, an ELISA assay was set up to detect EC-IgG immune-complexes, that were found in ECTM mothers' sera and milk.

We then sought to evaluate whether these EC-IgG immune-complexes were able to induce an active immune response in the pups. An initial indication of this came from a significant higher level of

IgM against neu and of neu-specific IgM^+ memory B cells in ECTM offspring over control. However, the proof of active immunization in breastfed pups came from the observation of an *in vivo* cytotoxic response against the neu p63-71 peptide in ECTM offspring. It is known that live activated leukocytes, including $CD8^+$ T cells, are present in mother's milk, that they can be transferred during breastfeeding and that they can enter pups' intestinal tract tissue and mesenteric LNs in some species [31]. It can be hypothesized, in the case of syngeneic strains, that these passively transferred cells may survive in the pups and be found in mesenteric LNs 5 weeks after birth, when the *in vivo* cytotoxicity assay was performed. Nevertheless, this possibility was ruled out by the Vβ-Jβ spectratype analysis results. We observed the expansion of $CD8^+$ T cells bearing the Vβ9-Jβ1.2 rearrangement in all ECTM mothers but in none of their neu^+ offspring; this Vβ-Jβ rearrangement is normally used as public in ECTM vaccinated BALB/c mice [21]. On the other hand, in 71% of neu^+ ECTM offspring there was expansion of low avidity $CD8^+$ T cells bearing the Vβ6-Jβ2.7 TCR rearrangement that is typical of ECTM vaccinated BALB-neuT, but not BALB/c, mice [22]. Indeed, we have recently shown how a temporary Treg depletion and ECTM vaccination in BALB-neuT mice was able to induce the expansion of latent pools of low-avidity $CD8^+$ T cells bearing TCR repertoires that react with p63-71 [22]. The Vβ6-Jβ2.7 rearrangement, the same expanded in neu^+ ECTM offspring in the present study, was among them. Preliminary data show a decrease in spleen-derived Treg percentage in neu^+ ECTM offspring as compared to neu^+ control offspring at the 5^{th} week of age (not shown). This may explain the presence of reactive, although low avidity, $CD8^+$ T cell clones in neu^+ ECTM offspring.

In conclusion, all together these findings are proof of concept of the efficacy of maternal immunization against an oncoantigen. We herein suggest that the concept of maternal immunization, being a potent weapon against pathogen-induced diseases in newborns, can be extended and used to delay cancer development in genetically predestinated offspring. The potential applications of this groundbreaking approach to neonatal cancer diseases such as neuroblastoma,

rhabdomyosarcoma, Wilms tumor and retinoblastoma may have a substantial impact on clinical practice.

Materials and methods

Mice. BKO mice [28], and FcγKO mice [30] were crossed with BALB-neuT mice to generate BALB-neuT/BKO and BALB-neuT/FcγKO mice, respectively. All mice were bred under specific pathogen-free conditions at the Molecular Biotechnology Center (Torino, Italy) and treated in conformity with European Guidelines and policies, as approved by the Ethical Committee of the University of Torino.

Cells. TUBO cells, an *in vitro* established neu^+ cell line derived from a lobular carcinoma arising in a BALB-neuT female mice [24], were cultured in DMEM supplemented with GlutaMAX™, D-glucose, HEPES buffer (Gibco), and 20% FBS (Sigma-Aldrich).

Immunization and tumor growth. The pCMV3.1 control, the ECTM [16] and pAmot [26] plasmids were generated as previously described. The pAAV-MCS (control plasmid) and the pAAV-MCS plasmid coding for Escherichia coli β-galactosidase (LacZ plasmid), were from the AAV Helper-Free System (Agilent Technologies Inc.). 50 μg of plasmids diluted in saline were injected into the quadriceps muscle of anesthetized mice. Immediately after injection, two 25-ms trans-cutaneous low voltage electric pulses (amplitude 150 V; interval 300 ms) were administered at the injection site via a multiple needle electrode connected to a Cliniporator™ (IGEA Srl). Female mice were immunized at 10 and 12 weeks of age and mated at week 13. All pups were fed by their own mother and weaned at 4 weeks of age. BALB-neuT female offspring mammary glands were inspected weekly for tumor appearance from the 12^{th} week of age. 5 week-old neu^- offspring were challenged subcutaneously in the inguinal region with $1x10^5$ TUBO cells. Tumor masses were measured as previously described [33].

Assessment of anti-neu and anti-Amot antibodies. Sera from mothers were collected 2 weeks after the last vaccination. Pups' sera were collected from 1 to 8 weeks after birth. Mothers were separated from their litters 3 weeks after delivery for milk collection and fed with hydrated food for

24 hours. 2 IU of oxytocin (PitocinaIniet; Farmaceutici Gellini Srl) were injected twice i.p. at an interval of 5 minutes between each administration. Milk was manually expressed from anesthetized mice, collected and mixed with a protease inhibitor cocktail (Sigma-Aldrich). Defatted milk was obtained after an initial room temperature centrifugation for 10 minutes, at 2.000 rpm and two subsequent 4°C centrifugations at 12.000 rpm for 90 minutes. Sera and milk samples were tested by ELISA. 96 well/plates (Costar®, Sigma-Aldrich) were coated with 100 ng/well of recombinant EC neu (Genway) or recombinant human-Amot (Origene) protein, overnight at 4°C. Coated plates were then blocked with 10% NCBS (Newborn Calf Serum; Sigma-Aldrich) in PBS (Invitrogen)-Tween (Sigma-Aldrich) 0.05% buffer for 2 hours at 37°C. Plates were incubated with samples diluted 1:100 in 1% blocking buffer for 1 hour at 37°C. Plates were washed 3 times with a PBS-Tween buffer. The HRP-conjugated anti-mouse IgG antibody (Sigma-Aldrich) (1:2000 dilution in blocking buffer) was incubated for 1 hour at 37°C. Plates were washed 6 times and chromogenic 3,3′,5,5′-Tetramethylbenzidine substrate was added (TMB; Sigma-Aldrich). The reaction was stopped by the addition of HCl 2N and optical density was measured at 450 nm using a microplate reader (680XR, BioRad). IgG isotype titration was performed using rat biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (BD Pharmingen) as secondary antibodies. Plates were then incubated for 30 minutes at room temperature with streptavidin-HRP (R&D Systems) diluted 1:200 in a PBS-Tween buffer and reactions were carried forward as described above.

Memory B cell ELISPOT. Splenocytes (SPC) from 5 week-old control and ECTM offspring were collected and stimulated with a mixture of R848 (1 µg/ml, Mabtech) and rmIL-2 (10 ng/ml, Mabtech). 72 hours later, B cells were isolated by positive immune selection after SPC incubation with a biotin-antibody cocktail against CD43 (BD Pharmingen) and CD11c (eBioscience) for 20 minutes at 4°C, followed by an incubation for 10 minutes at 4°C with Anti-Biotin MicroBeads (Miltenyi Biotec). $1x10^5$ B cells were plated in triplicate on PVDF ELISPOT plates (Mabtech) pre-coated with 20 µg/ml of recombinant EC neu protein. Plates were incubated for 24 hours at 37°C

and developed according to manufacturer's instructions (ELISpotPLUS, Mabtech). Specific spots were enumerated using the Transtec 1300 ELISPOT Reader (AMI Bioline). The number of specific spots was calculated by subtracting the number of spontaneously produced spots and expressed as spot-forming unit (SFU)/10^6 cells.

Immunoblotting. A total cell lysate from mouse cardiomyocytes which express β-galactosidase protein after recombinant Adeno-associated virus 2 viral particles (kindly provided by Prof. Emilia Turco, Molecular Biotechnology Center, University of Torino) infection, was separated by SDS-PAGE. Sera from vaccinated mothers and from their 3-week-old offspring were collected, pooled, diluted 1:50 in TTBS and incubated overnight at 4°C on the membrane, as primary antibody. Goat anti-mouse HRP secondary antibody (Sigma-Aldrich) was used for detection. Policlonal rabbit anti-β-galactosidase (Thermo Scientific) and anti-HSP90 antibody (Santa Cruz) were used as positive and loading control, respectively and detected by goat anti-rabbit HRP (Sigma-Aldrich). Proteins were detected by enhanced chemiluminescence (ECL®, Amersham Biosciences).

***In vivo* cytotoxicity assay.** 10^7 naive SPC/ml were labeled with two different concentrations (0.5 μM or 5.0 μM) of the fluorescent dye CFSE (Molecular Probes). 5 μM labeled-SPC were also pulsed with p63-71 (TYVPANASL; InBios Srl) for 90 minutes at 37°C. The two SPC populations were mixed in equal amounts and injected into the tail vein of mothers vaccinated with control or ECTM mothers two weeks after the last vaccination and into 5 week-old control or ECTM offspring. Forty-eight hours later, single-cell suspensions from the spleen of each mouse were processed to evaluate the presence of $CFSE^{high}$- and $CFSE^{low}$-labeled SPC on a CyAn ADP Flow Cytometer (DakoCytomation). The low peak percentage was normalized to control untreated low peaks and the specific cytolytic activity was calculated as: 100 – [($CFSE^{low}$ untreated cells / $CFSE^{low}$ experimental cells) x $CFSE^{high}$ experimental cells] x 100 / $CFSE^{high}$ untreated cells.

TCR repertoire analysis. Popliteal, inguinal and mesenteric lymph nodes (LNs) were collected from 5-week-old control and ECTM offspring and cultured for 3 days with or without 15 μM of p63-71. Total RNA was isolated from the recovered LNs cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized using an oligo-dT primer (dT15) (Invitrogen). cDNA was subjected to PCR amplification using a common Constant (C) β primer (CACTGATGTTCTGTGTGACA) in combination with the following Vβ primers: 6, CTCTCACTGTGACATCTGCCC; 9, TCTCTCTACATTGGCTCTGCAGGC. Using 2 μl of PCR product as a template, run-off reactions were performed with the following internal fluorescent Jβ: 1.2, AAAGCCTGGTCCCTGAGCCGAAG; 2.7, CTAAAACCGTGAGCCTGGTGC. Run-off products were denatured in formamide and analyzed on an Applied Biosystem 3100 Prism using Gene-scan 2.0 software (Applied Biosystem). Data were reported as the rate of stimulation index (RSI): normalized peak area from stimulated cells/normalized peak area of non-stimulated cells. T cells carrying a TCR rearrangement were considered to be expanded in a vaccination-driven manner when RSI was >2 [21].

EC-IgG immune complex detection. 96 well/plates (Costar®, Sigma-Aldrich) were coated with 200 ng/well of the anti-CD340 monoclonal antibody (Sino Biological Inc.), saturated with 5% NCBS in PBS-Tween 0.05 % for 1 hour at 37°C and, after several washes, the plates were incubated for 2 hours at 37°C with a 1:50 dilution of both milk and serum followed by HRP-conjugated anti-mouse IgG antibody. The following reactions were carried forward as described for the detection of anti-neu antibodies.

IFN-γ ELISPOT assay. $1x10^6$ SPC from 5-week-old control and ECTM offspring were plated in triplicate into nitrocellulose 96-well HTS IP plates (Millipore, Bedford, MA) which had been pre-coated with 5 μg/ml of rat anti-mouse IFN-γ antibody (clone R4-6A2, BD Biosciences). SPC were stimulated for 48 hours at 37°C with 15 μg/ml of p63-71. Plates were developed according to

manufacturer's instructions (BD^{TM} ELISPOT Set, BD Biosciences). Spots were enumerated as described above.

Statistical analysis. Statistical differences were evaluated using the GraphPad software 5.0 (GraphPad Inc.). The Mantel-Haenszel Log-rank test was used to analyze differences in the incidence of tumors, while the Student's *t* test was used for the evaluation of all other statistical differences.

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Figure Legends

Figure 1. DNA vaccine-induced anti-neu antibodies are successfully transferred from mothers to their pups and induce delayed mammary carcinoma onset in neu^+ offspring. (**A**) Detection of vaccination-induced anti-neu antibodies in the sera and milk of control (white bars) and ECTM (black bars) vaccinated mothers and in the sera of their 4 week-old offspring. **P = 0.004; ***P ≤ 0.0003, Student's *t* test. Data are representative of two independent experiments and represented as mean ± SEM. (**B**) Characterization of IgG subclasses of anti-neu antibodies in the sera and in the milk of ECTM mothers and in the sera of their 3-4 week-old offspring. (**C**) Detection of anti-neu IgG in control (white bars) and ECTM (black bars) offspring's sera collected from the 1^{st} to the 8^{th} week after birth. (**D**) Appearance of the first palpable mammary tumor in control (dotted black line, n = 12) and ECTM (continuous black line, n = 26) neu^+ offspring. Data are representative of four independent experiments. ***P < 0.0001, Mantel-Haenszel Log-rank test.

Figure 2. Maternal immunization against an oncoantigen, but not an unrelated antigen, delayed mammary carcinoma onset in neu^+ offspring. (**A**) Detection of vaccination-induced anti-Amot antibodies in sera and milk of control (white) and ECTM (black) mothers and in the sera of their 4 week-old offspring. ***P ≤ 0.0003, Student's *t* test. (**B**) Tumor incidence in control (dotted black line, n = 11) and pAmot (continuous black line, n = 18) neu^+ offspring. Data are representative of three independent experiments. **P = 0.001, Mantel-Haenszel Log-rank test. **(C**) Western blot analysis of β-galactosidase protein. Sera from control and LacZ mothers and their offspring were used as primary antibodies, recombinant β-galactosidase as positive control and HSP90 protein as loading control. (**D**) tumor incidence in control (dotted black line, n = 10) and LacZ (black line, n = 8) neu^+ offspring.

Figure 3. The presence of anti-neu antibodies and functional FcγRI/III are required to delay mammary carcinogenesis in neu$^+$ offspring. Tumor incidence of mammary carcinomas in neu$^+$ offspring of BKO (**A**) and FcγKO (**B**) control (dotted black lines; n = 7 BKO, n = 7 FcγKO) and ECTM (black lines; n = 6 BKO, n = 5 FcγKO) mothers. Data are representative of two independent experiments.

Figure 4. The passive transfer of maternal immunity induces an active humoral immune response in offspring. (**A**) Detection of EC-IgG immune-complexes in mothers' milk and sera. (**B**) Detection of anti-neu IgM in mothers' milk and sera and in the sera of their 3-4 week-old offspring. (**C**) Presence of neu-specific IgM$^+$ memory B cells in 5 week-old neu$^-$ and neu$^+$ offspring. Neu-specific IgM secreting cells are expressed as SFU/1x10^6 B cells. In all panels, white bars refer to control, black bars to ECTM. Data show the mean ± SEM of values obtained from two to three independent experiments. *P = 0.01; **P = 0.004; ***P ≤ 0.0007, Student's *t* test.

Figure 5. The passive transfer of maternal immunity induces an active cytotoxic immune response and the expansion of a distinct TCR repertoire in offspring. (**A**) *In vivo* cytotoxic response against p63-71 in control (white dots) or ECTM (black dots) mothers and in their neu$^-$ and neu$^+$ 5 week-old offspring. (**B**) TCR repertoires in ECTM vaccinated mothers and in their neu$^-$ and neu$^+$ offspring. Immunoscope analysis was performed on cDNA pools obtained from LNs of ECTM mothers (n = 3) and their neu$^+$ (n = 7) and neu$^-$ (n = 5) 5 week-old offspring. LNs cells were re-stimulated *in vitro* with the p63-71. Vβ9-Jβ1.2 and Vβ6-Jβ2.7 rearrangement frequencies are shown. Data are representative of two independent experiments. (**C**) T-cell response against p63-71 was quantified *in vitro* with an IFN-γ-based ELISPOT assay. IFN-γ-producing cells from control (white bars) and ECTM (black bars) neu$^-$ and neu$^+$ offspring are expressed as SFU/1x10^6 SPC. *P = 0.01; **P = 0.005; ***P = 0.0008, Student's *t* test. Graphs display mean ± SEM and are representative of two independent experiments.

Table 1. Maternal immunization against neu hampers the growth of a transplantable mammary tumor

	Tumor takes/challenged mice	Latency time (days)[a]				Survival time (days)[a]
		2 mm[b]	4 mm	6 mm	8 mm	10 mm
Control offspring	22/22 (100%)[c]	12.1 ± 0.6	15.5 ± 0.6	18.9 ± 1	22.5 ± 1.2	26.8 ± 1.3
ECTM offspring	20/22 (91%)	14.5 ± 0.8[*]	19.8 ± 0.7[***]	24 ± 0.6[***]	28.6 ± 1.1[***]	34.8 ± 1.4[***]

[a] The time taken by TUBO cells to give rise to tumors with a mean diameter of 2, 4, 6 and 8 (latency times) or 10 (survival time) mm. Data are representative of three independent experiments and are expressed as mean ± SEM

[b] mean tumor diameter

[c] percentage of survival in parentheses

[*] $P = 0.02$, [***] $P \leq 0.0006$ as compared to control offspring, Student's *t* test

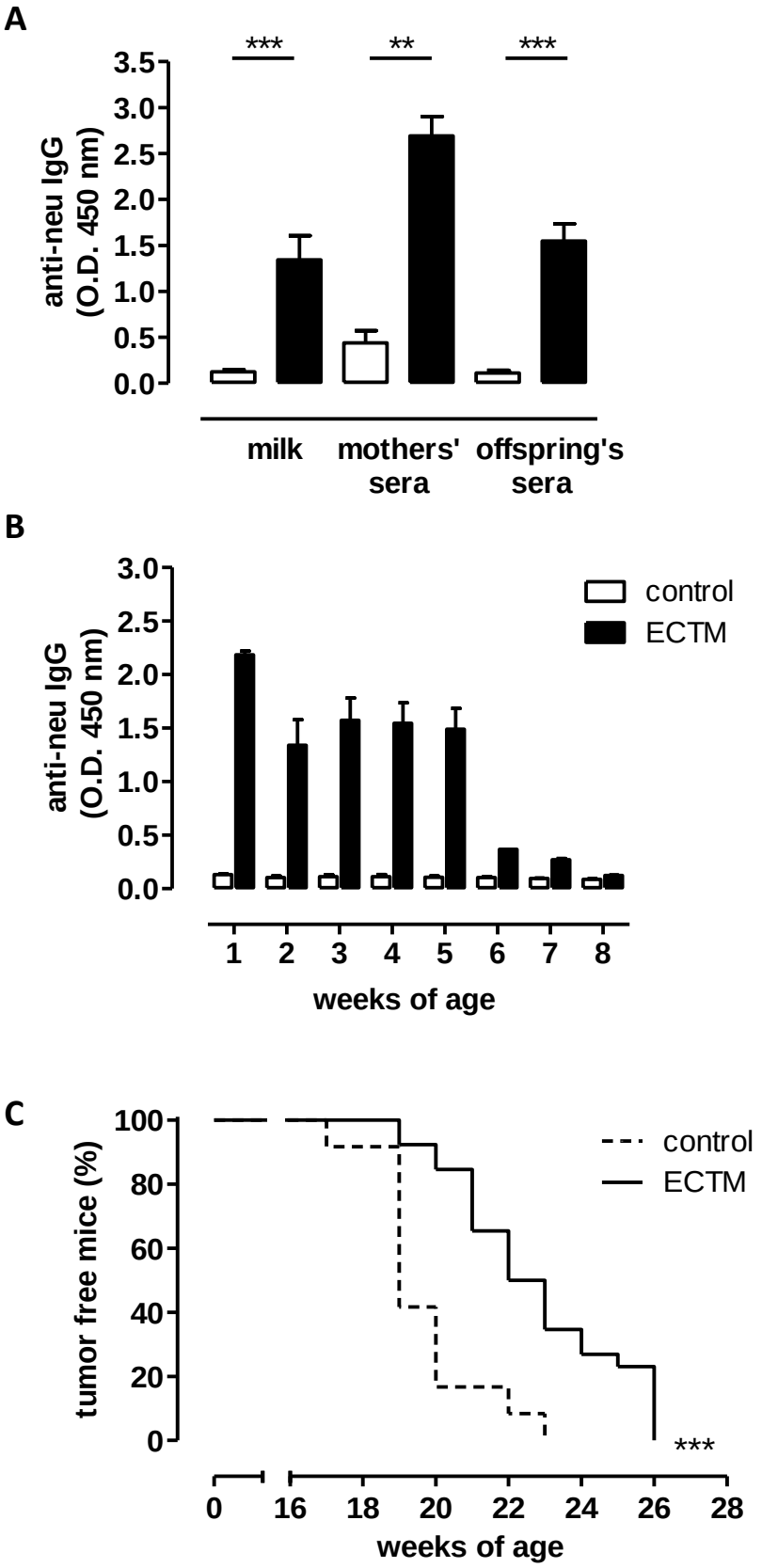


Figure 1, Barutello et al.

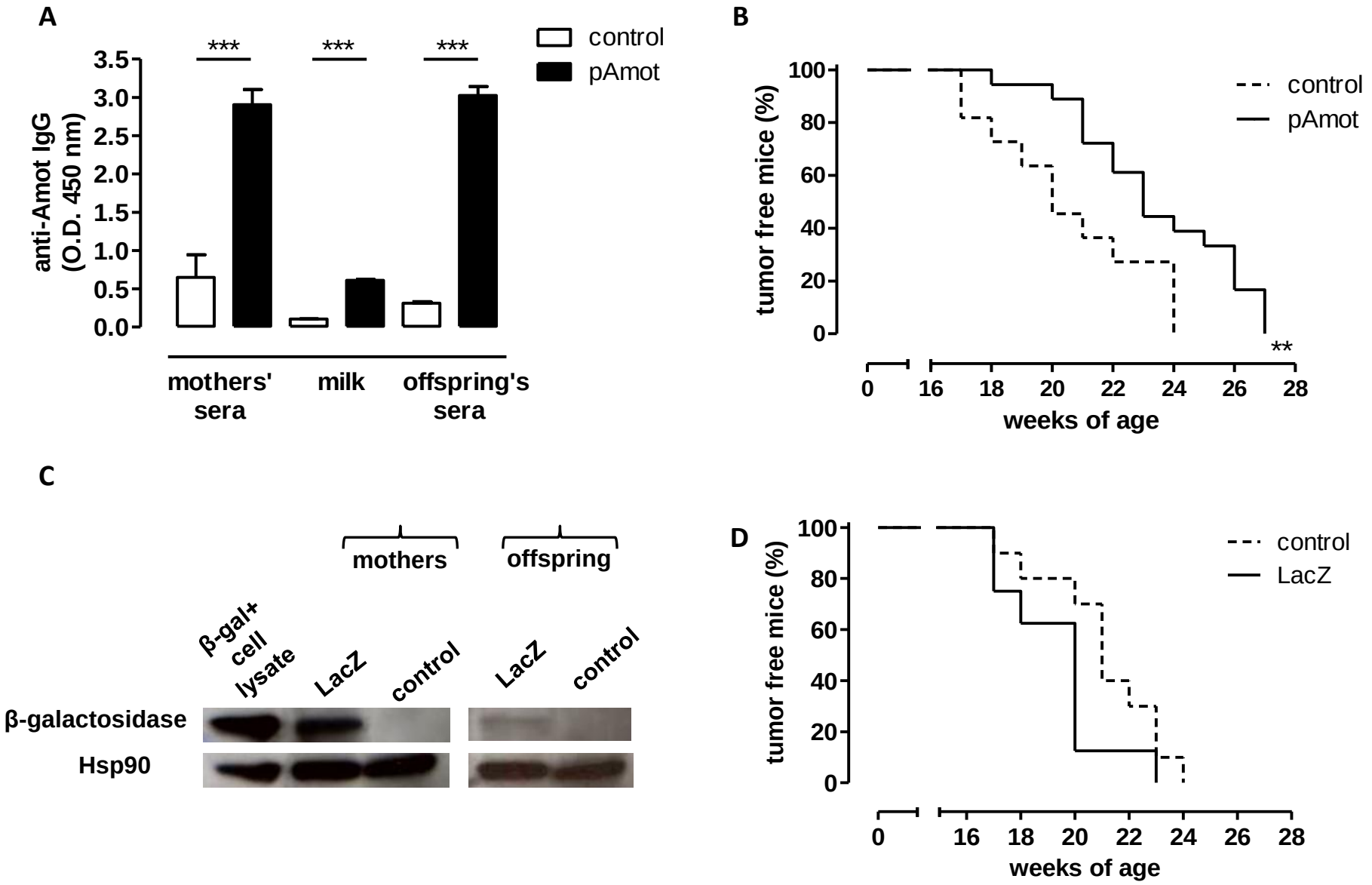
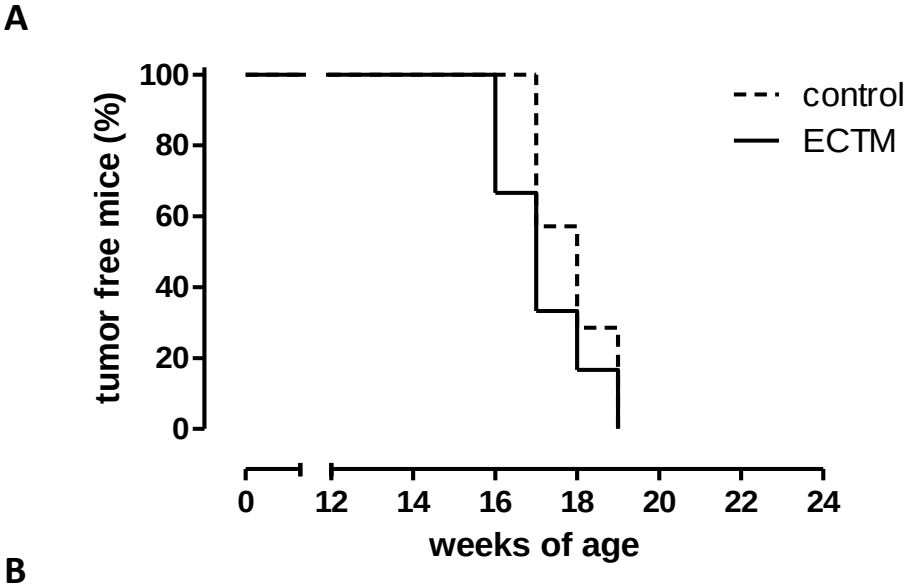
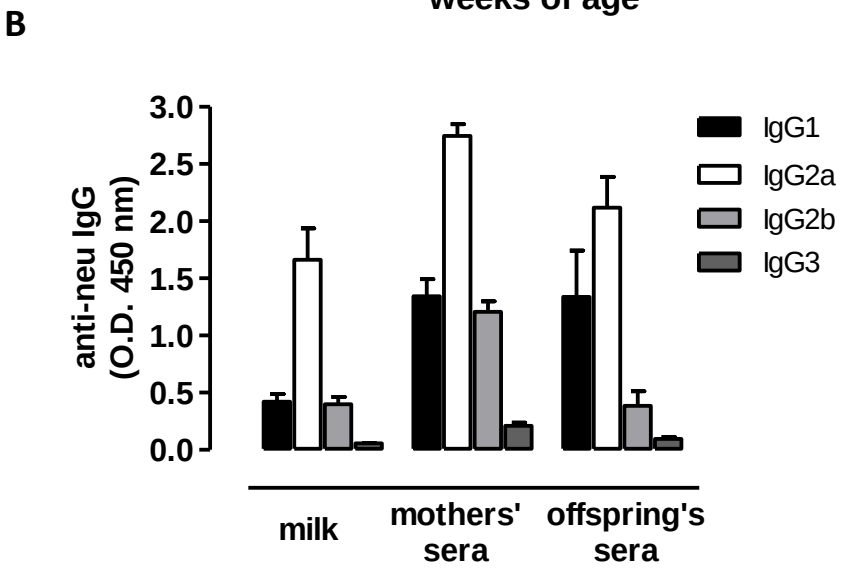


Figure 2,
Barutello et al.





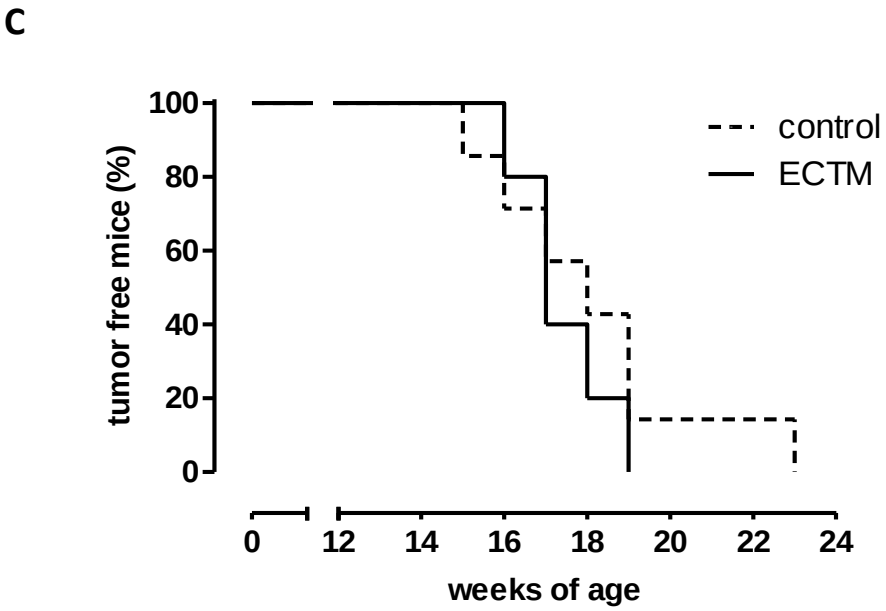


Figure 3,
Barutello et al.

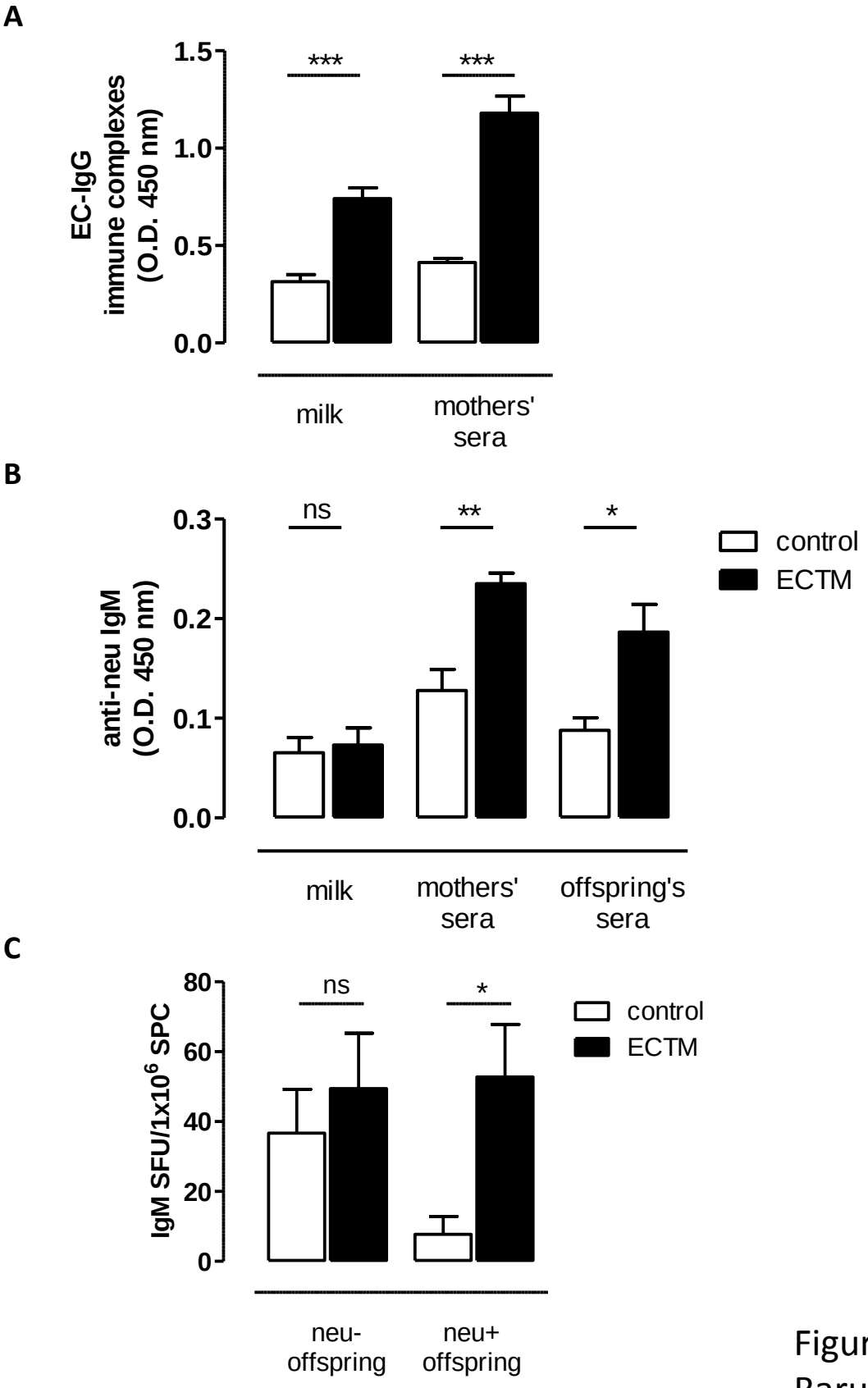
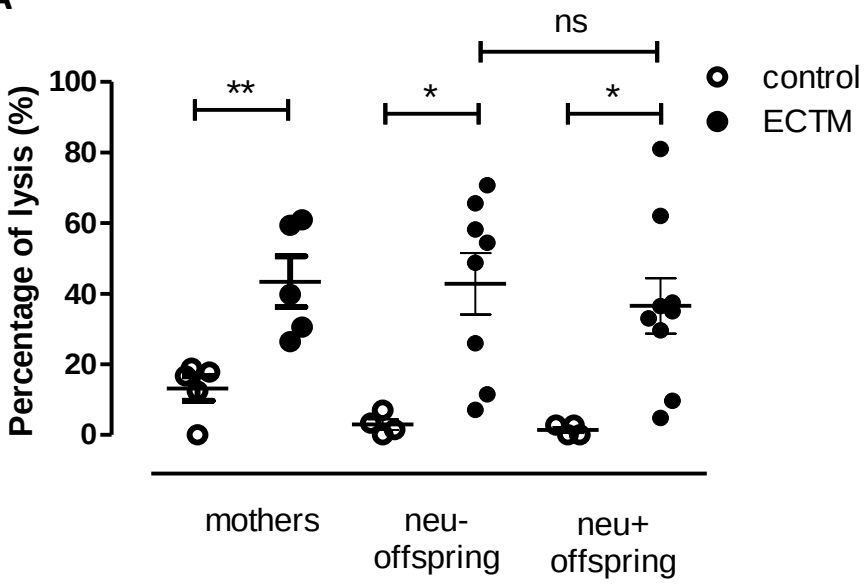


Figure 4,
Barutello et al.

A



B

	Vβ9-Jβ1.2	Vβ6-Jβ2.7
mothers	3/3	0/3
neu- offspring	3/5	0/5
neu+ offspring	0/7	5/7

C

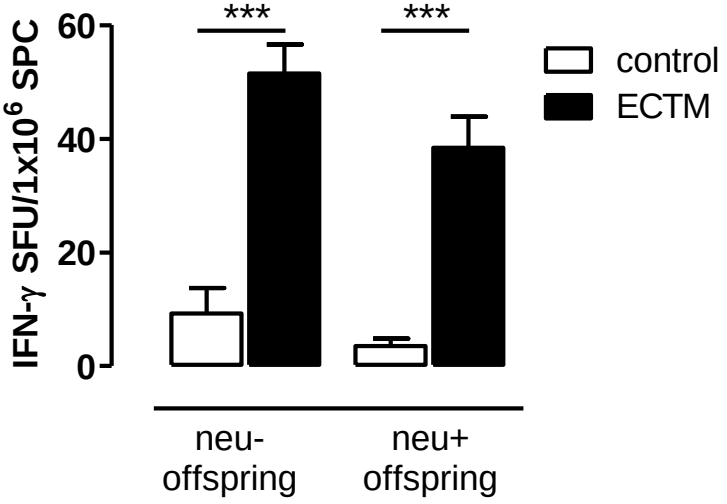


Figure 5,
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