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Supplementary Materials for

Mucosal Imprinting of Vaccine-Induced CD8$^+$ T Cells Is Crucial to Inhibit the Growth of Mucosal Tumors

Federico Sandoval, Magali Terme, Mevyn Nizard, Cécile Badoual, Michel-Francis Bureau, Ludovic Freyburger, Olivier Clement, Elie Marcheteau, Alain Gey, Guillaume Fraisse, Cécilia Bouguin, Nathalie Merillon, Estelle Dransart, Thi Tran, Françoise Quintin-Colonna, Gwennhael Autret, Marine Thiebaud, Muhammad Suleman, Sabine Riffault, Tzyy-Choou Wu, Odile Launay, Claire Danel, Julien Taieb, Jennifer Richardson, Laurence Zitvogel, Wolf H. Fridman, Ludger Johannes, Eric Tartour*

*To whom correspondence should be addressed. E-mail: eric.tartour@egp.aphp.fr

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The PDF file includes:

Flow cytometry analysis

To detect anti-$OVA_{257–264}/K^b$- or anti-$E7_{49–57}/D^b$-specific $CD8^+$ T cells, cells were purified by anti-CD8 microbeads (Miltenyi Biotec). Purified $CD8^+$ T cells from the spleen, mediastinal or cervical lymph node cells obtained 7 days after the second immunization were stained with $OVA_{257–264}/K^b$ or $E7_{49–57}/D^b$ tetramer, according to the manufacturer's recommendations (Beckman Coulter Immunomics). Briefly, cells were incubated at 4°C with the PE-labeled tetramer for 35 minutes. After incubation and washes, labeled anti-CD8 mAbs (eBioscience) were used to phenotype the positive tetramer $CD8^+$ T cells. Irrelevant tetramers recognizing a vesicular stomatitis virus (VSV) or LCMV derived peptide in the context of K^b or D^b molecules were used in each experiment. Naive non immunized mice were also included as a control for these experiments. To detect tumor infiltrating anti-$E7_{49–57}/D^b$-specific $CD8^+$ T cells, tumors were dissociated and then washed twice in PBS and incubated with the Fc receptor block CD16/CD32 (eBioscience). They were then labeled with the PE-tetramer and the anti-CD8 antibody. Expression of the $K^bOVA_{257–264}$ complex by purified mediastinal lymph node $CD11c^+$ cells (CD11c N418 MicroBeads Miltenyi Biotec) was monitored by staining with the 25-D1.16-APC conjugated mAb (eBioscience) and CD11c-PE mAb (eBioscience) and CD11b PE-Cy7 (Biolegend) seven days after i.n. vaccination with STxB-OVA or OVA mixed with αGalCer.

For the analysis of the expression of integrins and chemokine receptors, tetramer positive $CD8^+$T cells were co-stained with anti mouse CD103 Pacific Blue mAb (Biolegend), anti mouse CD49a Alexa 647 mAb (clone Ha31/8) or purified NA/LE hamster anti rat/mouse CD49a washed and stained with an FITC mouse anti Armenian and Syrian hamster IgG cocktail (BD Pharmingen), Alexa-488 anti mouse CCR5 mAb (Biolegend), APC anti mouse CXCR6 mAb (R&D, Lille. France), PercP

Cy5.5 anti mouse CXCR3 mAb (Biolegend), FITC anti mouse CD49d (eBioscience), APC anti mouse α4β7 mAb (eBioscience) and Live/Dead (Invitrogen) to exclude dead cells.

The biotinylated anti-mouse E-cadherin was purchased at Abcam.

Analyses were done in a BD Biosciences LSR II or BD Biosciences FACSCalibur with FlowJo (Tree Star) or Cell Quest software (BD Biosciences).

DC co-cultured with OT-1 cells

B6 mice were i.n. immunized or not with STxB-OVA + αGalcer. Two hours after vaccination, mice were sacrificed and the lungs were removed and treated with DNase I (30UI/mL) Collagenase D (1mg/mL) (Roche) for 1h at 37°C then passed through a 40µm cell strainer (BDBiosciences). Cells were washed in PBS-EDTA 2mM, centrifuged then re-suspended and red blood cells were lysed by adding ACK buffer for 2min then washed and re-suspended. Cells were counted, pre-incubated with anti-CD16/CD32 (eBioscience) and stained with an anti-mouse $CD11c^+$ PE mAb (eBioscience clone N418). $CD11c^+$cells were then isolated by flow sorting (FACSARIA II, BD Biosciences). Dead cells were excluded by Live/Dead (Invitrogen) staining. Spleens from OT-I mice harbouring a transgenic TCR recognizing the K^b-$OVA_{257-264}$ complex (Charles River) were passed through a cell strainer then washed and $CD8^+$ T cells were purified by anti-CD8 microbeads (Miltenyi Biotec). A total of 2 × 10^4 DCs and 2 x 10^5 OT-I cells were co-cultured in 200µL of complete RPMI (GIBCO) medium for 72h. Cells were recovered and washed twice in PBS then pre-incubated with anti-CD16/CD32 (eBioscience) and stained with an anti-mouse CD49a Alexa 647 mAb (clone Ha31/8 BD Biosciences), anti-mouse CD8 FITC mAb (eBioscience) and Live/Dead to exclude dead cells.

MRI images

MRI images were acquired on the 4,7T BrukerBiospec system (Bruker) of the Paris-Descartes Small Animal Imaging facility, using a 35mm diameter quadratic resonator. Coronal T2-weighted images of the head of the mouse were acquired after positioning. The imaging parameters of the TurboRARE-T2 sequence were as follows: repetition time (TR) / echo time (TE) = 2424ms/40ms, slice thickness = 1 mm, 15 slices, matrix size = 192 $\times$ 192 and field of view (FOV) = 25 $\times$ 25mm, which gave a resolution plan of 130 $\times$ 130μm and the acquisition time was 3min 4s. Mice were imaged 14 days after the tumor challenge. Tumor volume was determined in 3 dimensions with ImageJ software after defining a ROI of the tumor.

In vivo optical imaging of tumor cells luciferase activity

The substrate of luciferase, the luciferin potassium salt (SYNCHEM) diluted in PBS was injected intraperitoneally at a dose of 3mg/300µl. Optical imaging was performed with a cooled intensified charge-coupled device (CCD) camera (Biospace, Photo Imager) placed in a black box. Luminescence level was measured in a region of interest (ROI) corresponding to the chest. We used the same ROIs for all the mice analyzed. We opted to take the mean values in cpm of all the measurements for 20min after the start of acquisition.

SUPPLEMENTARY FIGURES

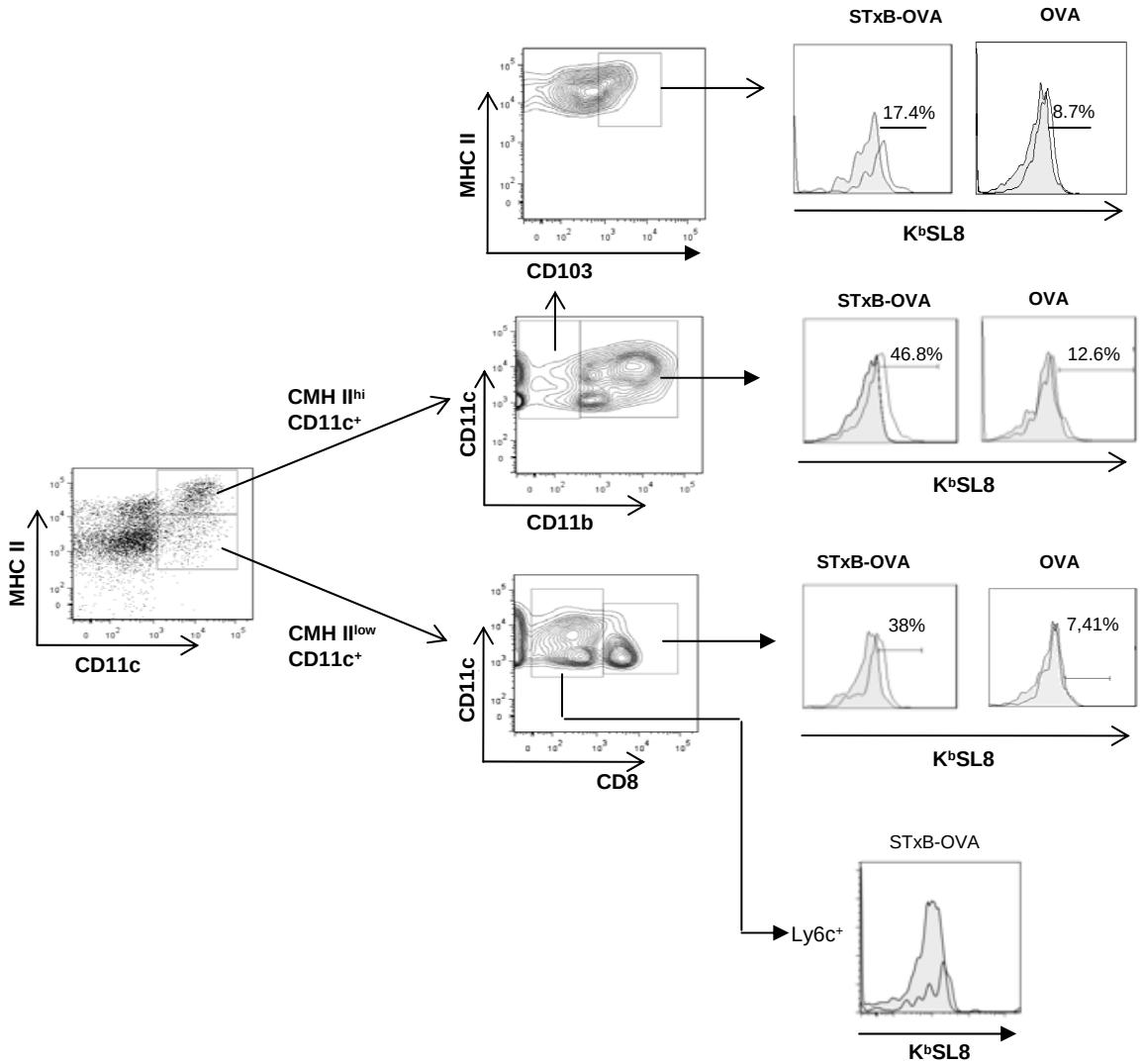

Figure S1 : Intranasal STxB-based vaccines target mediastinal DCs.

Mice were immunized two times (d0 and d14) by the i.n route with STxB-OVA (0.5 nmol) or OVA (0.5 nmol) mixed with αGalCer. Seven days after the second immunization mediastinal lymph nodes were collected and purified CD11c+ cells were costained with a mix of mAb (anti-MHC II, anti-CD11b, anti-CD8, anti-CD103, anti-Ly6c) to define the subpopulations of DC and the 25.D1.16 mAb (anti-K^b-$OVA_{257-264}$) and analyzed by cytometry. Isotype control antibodies were included in each experiment which were reproduced three times.

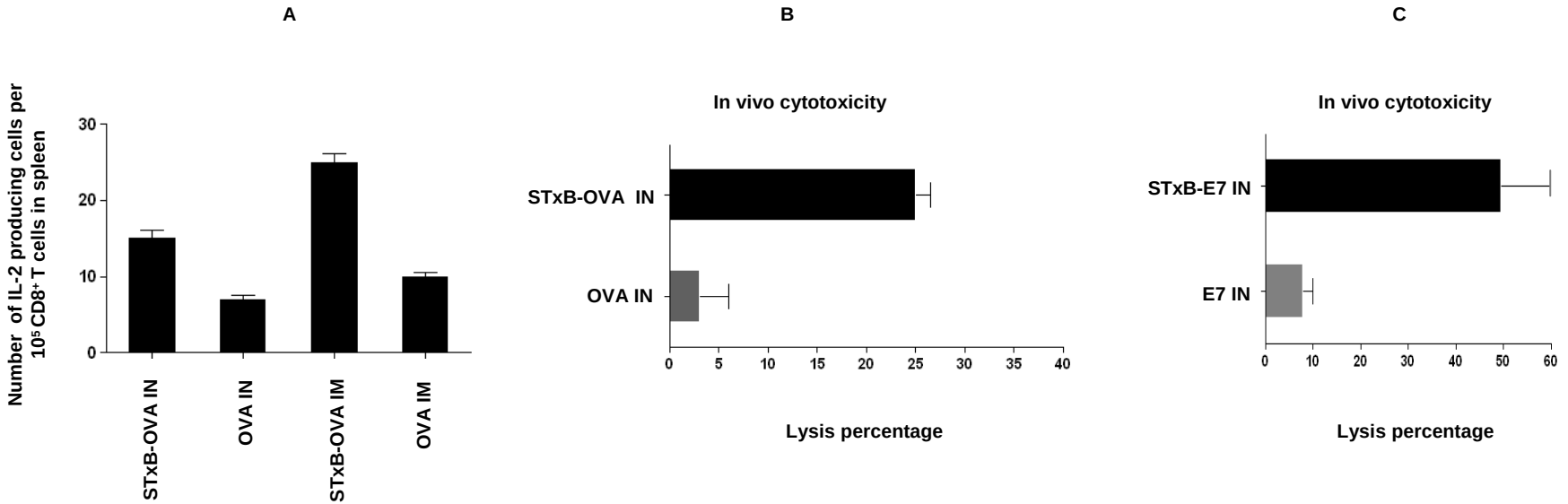

Figure S2 : Intranasal immunization with STxB induces multifunctional antigen-specific CD8$^+$ T cells.

After two i.n or i.m immunizations with OVA or STxB-OVA, ex vivo $OVA_{257-264}$ specific $CD8^+$ T cells producing IL-2 were detected by Elispot in the spleen (**a**). Splenocytes from naive B6 mice were labeled with 5μM CFSE ($CFSE^{high}$) and pulsed with $OVA_{257-264}$ (b) or $E7_{49-57}$ (c) at 10 μg/ml. Splenocytes labeled with 1μM CFSE ($CFSE^{low}$) served as non-pulsed target cells. CFSE-labeled cells containing an equal number of cells from each fraction (high and low) were injected into mice previously i.n immunized with OVA or STxB-OVA (b) or STxB-E7 or E7-derived peptide vaccines (c). After 15-18 h, single-cell suspensions from spleens were analyzed by flow cytometry. The percentage of specific killing was determined according to the following calculation: [1 - [($CFSE^{low}$ / $CFSE^{high}$ of naive mice) / ($CFSE^{low}$ / $CFSE^{high}$ of vaccinated mice)] x100.

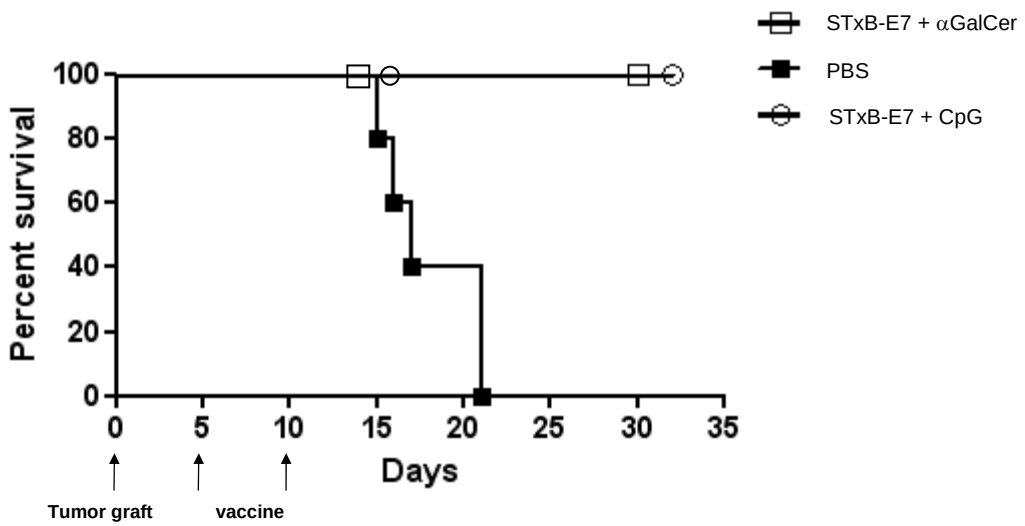

Figure S3 : STxB-E7 combined with CpG is efficient to inhibit mucosal tumor growth in a therapeutic setting.
In a therapeutic setting, mice were immunized via the i.n route with STxB-E7 mixed with αGalCer or CpG, 5 and 10 days after grafting TC1 cells into the submucosa of the tongue. As control, a group of mice were not immunized (PBS). Kaplan-Meier survival curves are shown. Experiments were repeated twice with 5 mice per group.

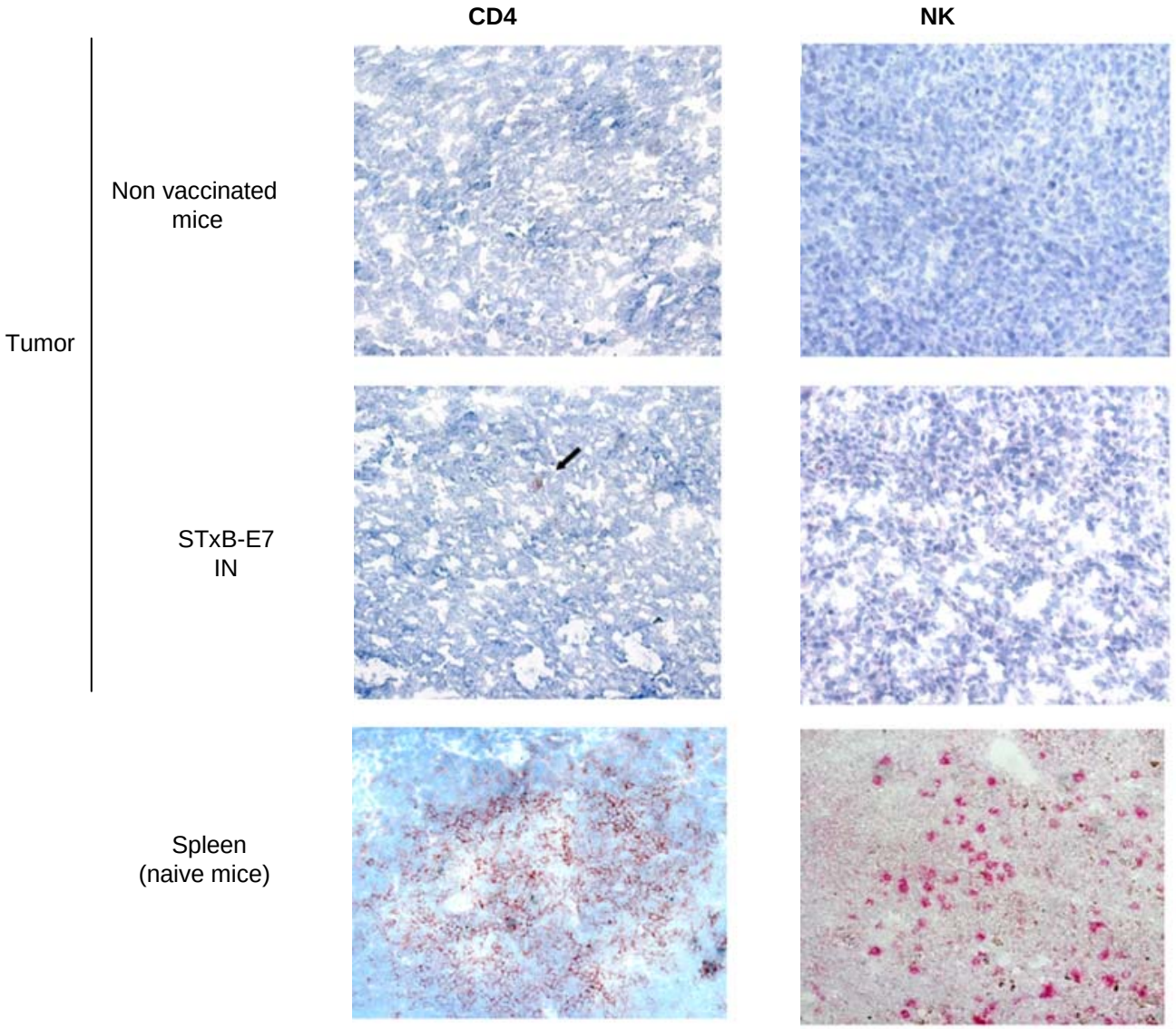

Figure S4 : CD4⁺ T cell and NK infiltration in mice vaccinated by STxB-E7 by the intranasal route.
TC1 were grafted in the submucosae of the tongue and mice were either immunized or not with STxB-E7 mixed with αGalCer, 5 and 10 days after tumor implantation. Tumors were collected 15 days after TC1 graft. NK and CD4⁺T cells were stained by immunocytochemistry. Spleen from naive mice was used as positive control. Isotype controls were also included in each experiment.

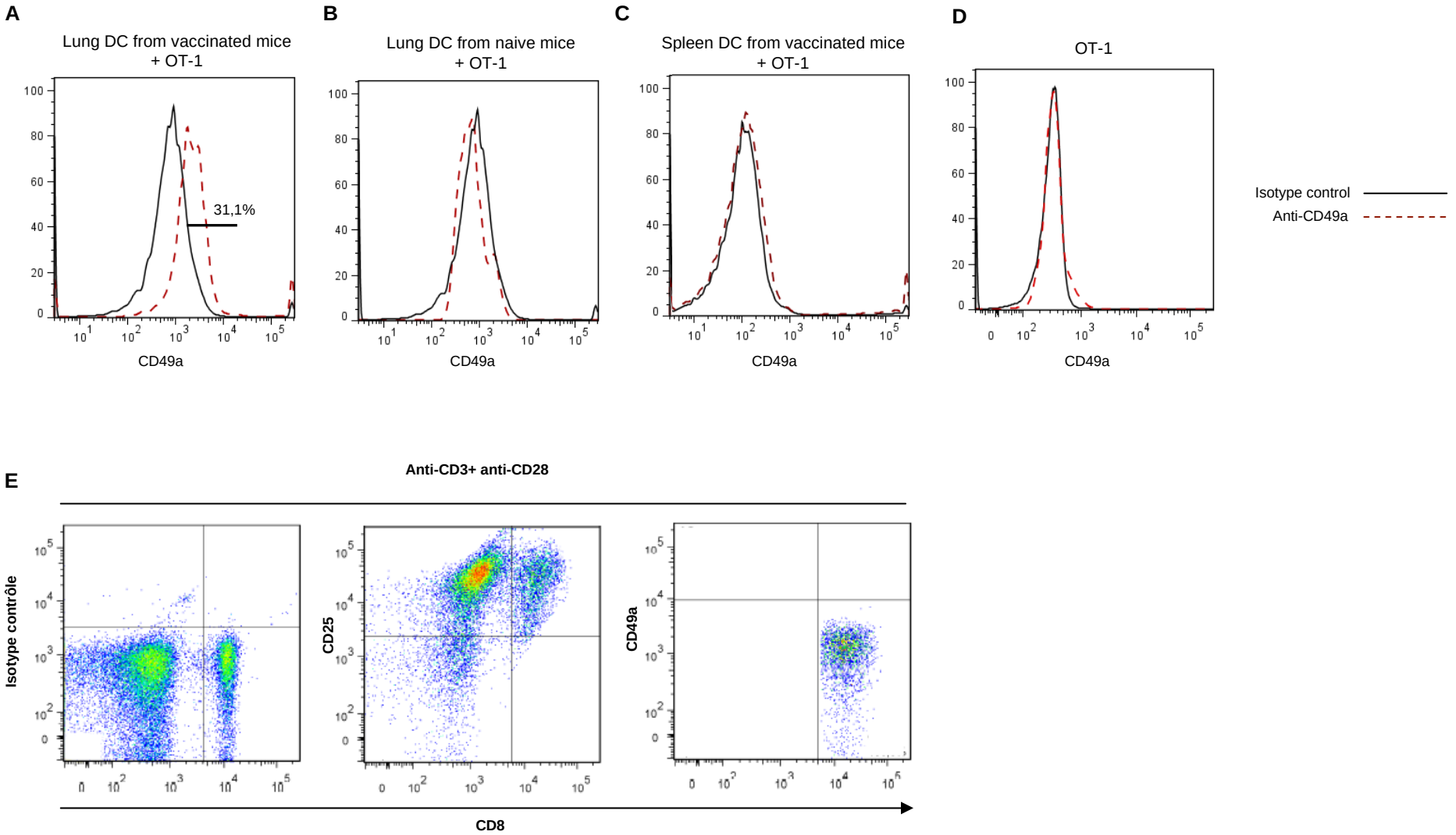

Figure S5 : Lung DCs induce the expression of CD49a on CD8⁺ T cells.

The expression of CD49a was evaluated by flow cytometry on A) OT-1 cells co-cultured in vitro for 72h in presence of sorted CD11c⁺ lung parenchyma DCs collected 2 hours after i.n immunization with STxB-OVA. B) OT-1 cells co-cultured for 3 days with sorted lung parenchyma DC from naïve mice. C) OT-1 cells co-cultured in vitro for 72h in presence of sorted CD11c⁺ DCs from splenocytes collected 2 hours after i.n immunization with STxB-OVA. D) OT-1 cells alone Each group included 10 mice and these experiments were reproduced three times. e) Splenocytes were activated by anti-CD3 and anti-CD28 and then stained for CD25, CD8 and CD49a expression.

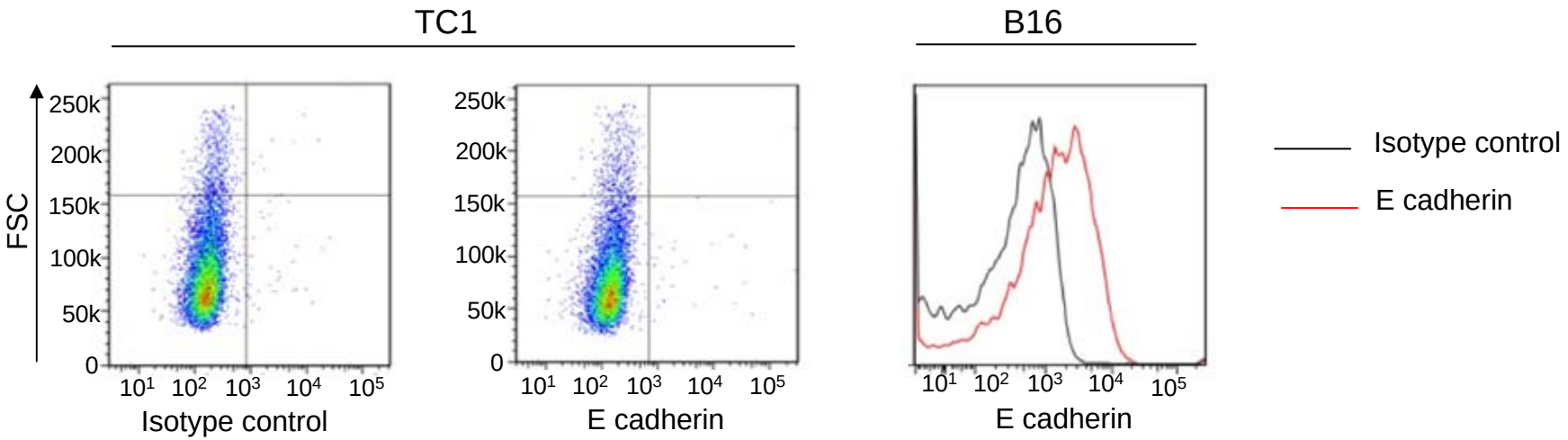

Figure S6 : TC1 cells do not express E-cadherin.

TC1 were stained with anti-E-cadherin or isotype control antibodies. B16 melanoma cells were also included as positive control in the staining experiment. Cells were then analyzed by cytometry

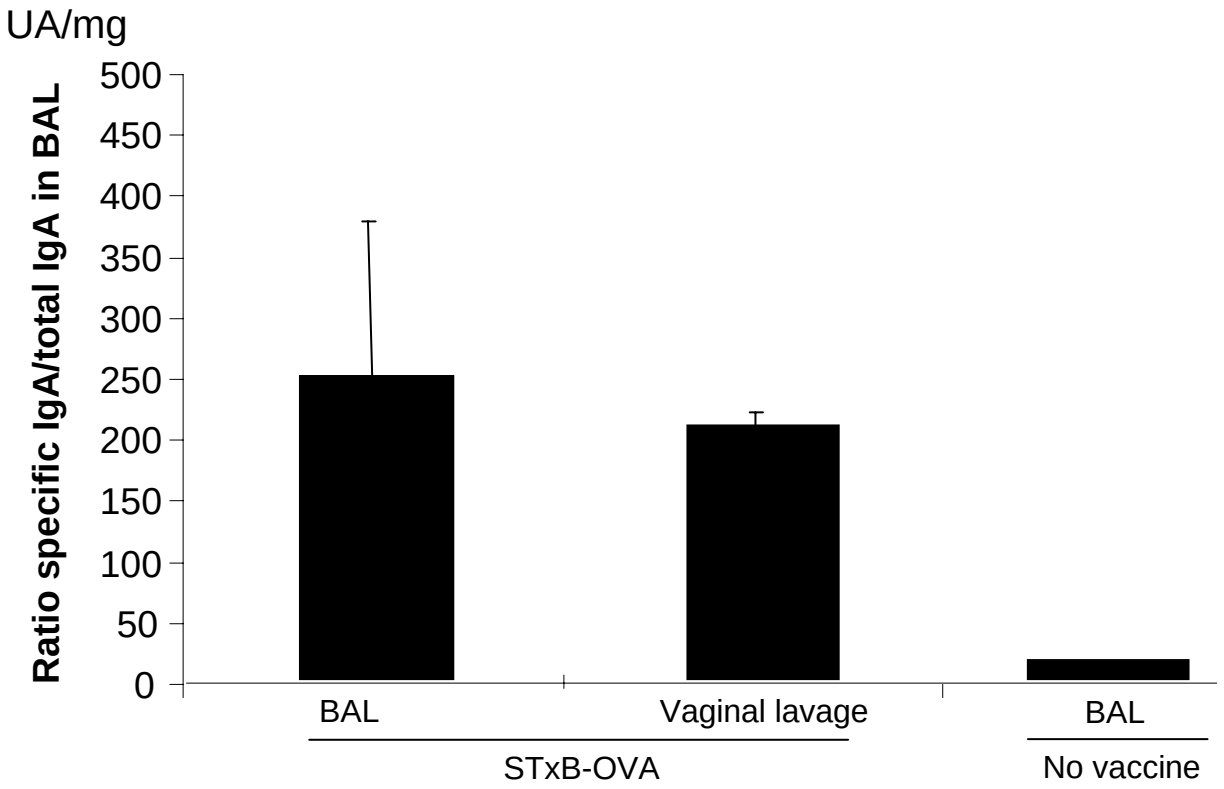

Figure S7 : Detection of specific IgA response in the BAL and vaginal lavage after intranasal immunization with STxB-OVA.
Mice were immunized or not immunized via the intranasal route at d0 and d14 by STxB-OVA (20 μg) mixed with αGalcer (1 μg). At day 21, total IgA and anti-OVA IgA were measured in BAL and vaginal lavage.