SUPPLEMENT

Title

Depletion of major pathogenic cells in asthma by targeting CRTh2

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Supplementary Material and Methods

Depletion studies in hCRTh2.BAC.Tg mice

Mouse or humanized anti-hCRTh2 antibodies or isotype control antibodies (mIgG2a: anti-ragweed antibodies; hIgG1: anti-gD antibodies) were intravenously injected on day 0 into 8-10 week old hCRTh2.BAC.Tg mice at doses indicated. Eye bleeds were taken on days indcated to analyze peripheral blood basophil and eosinophil numbers by flow cytometry. Alternatively, a group of mice were sacrificed on days indicated and blood, spleen and bone marrow were harvested and processed for enumeration of eosinophils and basophils by flow cytometry. Red blood cells were lysed with EL buffer (Qiagen). White blood cells, splenocytes or bone marrow cells were stained with anti-CD123, anti-FcεRI, and anti-CCR3 to detect basophils and eosinophils. Absolute cell numbers was determined by flow cytometry using CaliBRITE FITC beads (BD Biosciences).

Flow cytometry

To determine CRTh2 expression on regulatory T cells, CD4+CD25+ T cells were enriched from human PBMCs by MACS isolation (Miltenyi Biotec) and surface stained with anti-CRTh2, anti-CD4, and anti-CD25 followed by intracellular staining with anti-FoxP3 (BD Biosciences).

Dual-immunofluorescence confocal microscopy

To examine CRTh2 expression on human tissue mast cells dual immunofluorescence was performed on FFPE human tissue sections after antigen retrieval with EDTA pH 8.0 solution (ThermoFisher Scientific) using mouse anti-Tryptase mAb clone AA1 (Dako) in

parallel with isotype control naïve mouse clone MOPC-31C (BD Pharmingen) with biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch), followed by Vectastain ABC-HRP and Alexa Fluor 488 Tyramide (ThermoFisher Scientific). A second round of antigen retrieval to remove the antibody-HRP complex but not the Alexa Fluor 488 was then performed. Sections were subsequently incubated with rabbit anti-CRTh2 clone 108.1.6 (validated in separate experiments and showing comparable immunoreactivity to clone 81.12.4), in parallel with naïve rabbit monoclonal clone DA1E (Cell Signaling Technology), followed by goat anti-rabbit biotinylated secondary (VectorLabs) and Vectastain ABC-HRP, then detected with Alexa Fluor 594 Tyramide (ThermoFisher Scientific). All sections were counterstained with DAPI (ThermoFisher Scientific) and coverslipped with Prolong Gold (ThermoFisher Scientific). Images were captured on a Leica SPE confocal microscope equipped with 100x and 63x oil objective lenses (Leica Microsystems). Digital magnification, image compression, and merging were performed in Adobe Photoshop CS3.

Histology

Immunohistochemistry using rabbit anti-CRTh2 monoclonal antibodies, clone 81.12.4, was performed on FFPE human or mouse tissue using Target Retrieval pH 9.0 Solution (DAKO) for antigen retrieval as described in Material and Methods of the manuscript. Gastric corpus lamina propria eosinophils were blindly enumerated using sirius red staining across ten continuous high power (400x) microscopic fields (hpf) immediately adjacent to the forestomach mucosa.

Tissue	Number of patients immunoreactive – tissue microarray cores	Number of patients immunoreactive – tissue sections	Immunoreactive leukocyte distribution
Spleen	3 of 3	3 of 3	Red pulp, outer white pulp
Tonsil	3 of 3	3 of 3	Interfollicular zone
Thymus	1 of 3	3 of 3	Cortical zone
Lymph Node	na	3 of 3	Interfollicular zone
Asthmatic Airway	na	20 of 20	Submucosa
Stomach	2 of 3	3 of 3	Lamina propria
Small Intestine	2 of 3	3 of 3	Lamina propria
Colon	3 of 3	1 of 3	Lamina propria
Skin	3 of 3	na	Dermal perivascular zone
Uterus	2 of 3	na	Lamina propria

Supplementary Table 1

Summary of immune cell αnti-CRTh2 IHC immunoreactivity findings in normal human tissues and endobronchial biopsies of asthmatics. Normal human tissue microarraray cores (TMA) and select individual human tissue sections were stained with anti-hCRTh2 (clone 81.12.4) to evaluate for the presence and distribution of CRTh2+ immune cells.

Tissue	Number of unique patient tissues examined	Number of tryptase+ cells showing CRTh2 co-staining (>50 tryptase+ cells evaluated)
Allergic nasal polyp	13	0
BOBCAT asthma lung biopsies	10	0
Inflamed bronchus	1	0
Asthma lung	5	0
Normal stomach	1	0
Thymoma	1	0
Bone marrow	9	1 (faint)

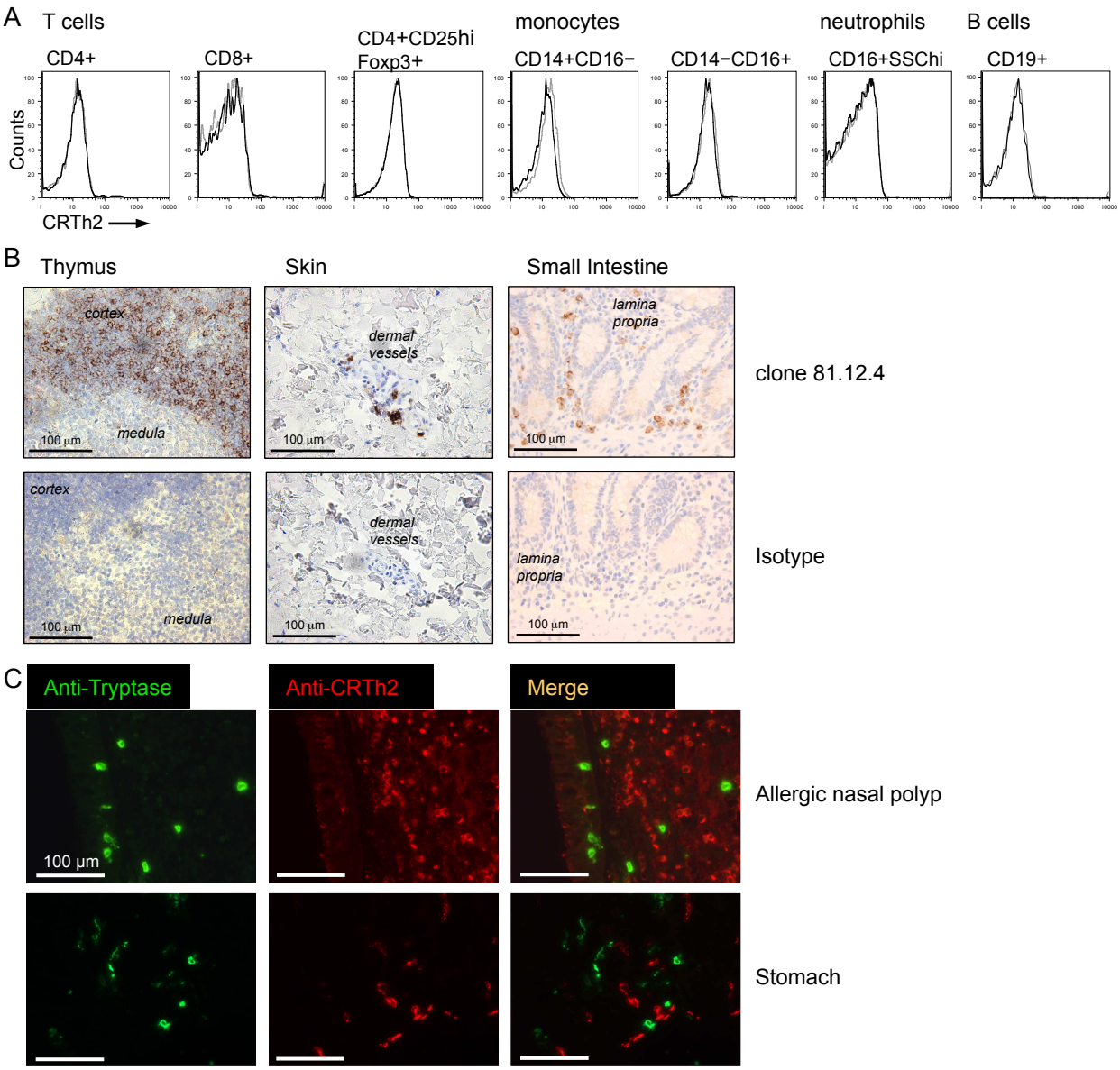
Supplementary Table 2

Summary of anti-Tryptase and anti-CRTh2 double immunofluorescence immunoreactivity findings in normal human tissues and biopsies of asthmatics or allergic patients. Normal human tissue sections or biopsies were double-stained with anti-hCRTh2 (clone 81.12.4 and/or clone 108.1.6) and the mast cell marker anti-Tryptase (clone AA1) to evaluate for the co-localization of CRTh2+ immune cells and mast cells. All isotype control staining was negative (not shown).

Gene Symbol	ABI_Assay_ID
Ccl2	Mm00441242_m1
Ccl3	Mm00441259_g1
Ccl8	Mm01297183_m1
Ccl11	Mm00441238_m1
Ccl17	Mm01244826_g1
Ccl24	Mm00444701_m1
Actb	Mm01205647_g1
Gapdh	Mm02758991_g1
Hprt1	Mm01545399_m1
Rpl19	Mm02601633_g1

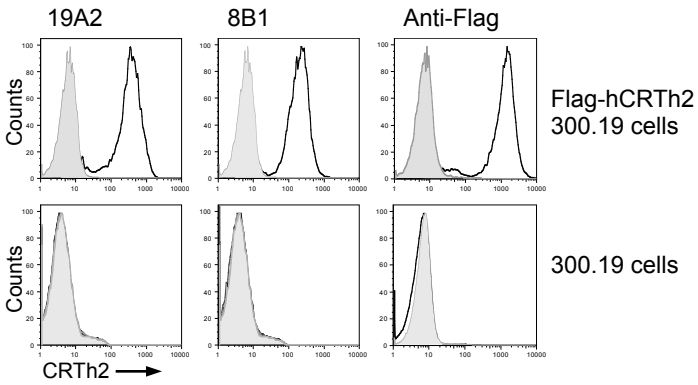
Supplementary Table 3

TaqMan Fluidigm probe sets.



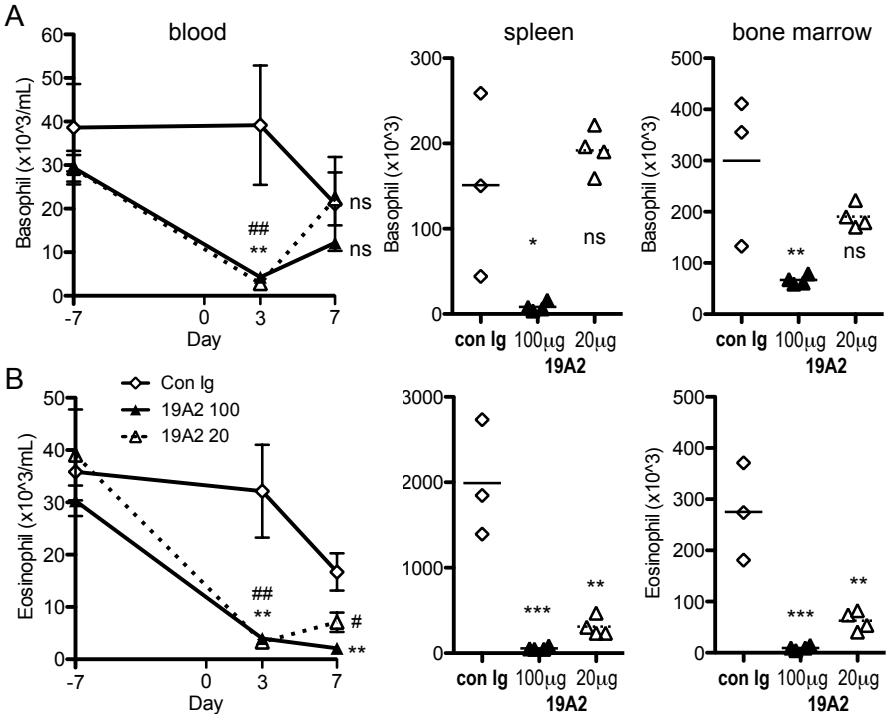
Supplementary Figure 1

Profiling of CRTh2 expression on human immune cells from PBMC and in select human tissues. (**A**) Flow cytometry analyses of CRTh2 expression on CD4+ or CD8+ T cells, T regulatory cells (CD4+CD25hiFoxp3+), monocytes (CD14+CD16−, CD14−CD16+), neutrophils (CD16+SSChi) or B cells (CD19+) from human peripheral blood leukocytes; anti-CRTh2 (clone BM16, black line), isotype control Ab (grey line) (**B**) Expression of CRTh2 in additional human tissues examined by immunohistochemistry using rabbit anti-hCRTh2 monoclonal Ab (clone 81.12.4; top panels) or isotype control antibody (lower panels). (**C**) Dual immunofluorescence confocal microscopy indicates that CRTh2 is not expressed on human typtase-positive mast cells in human allergic nasal polyps and stomach. Representative tissue images stained for the mast cell tryptase (clone AA1, green channel) and hCRTh2 (clone 108.1.6, red channel). Scale bars, 100 µm.



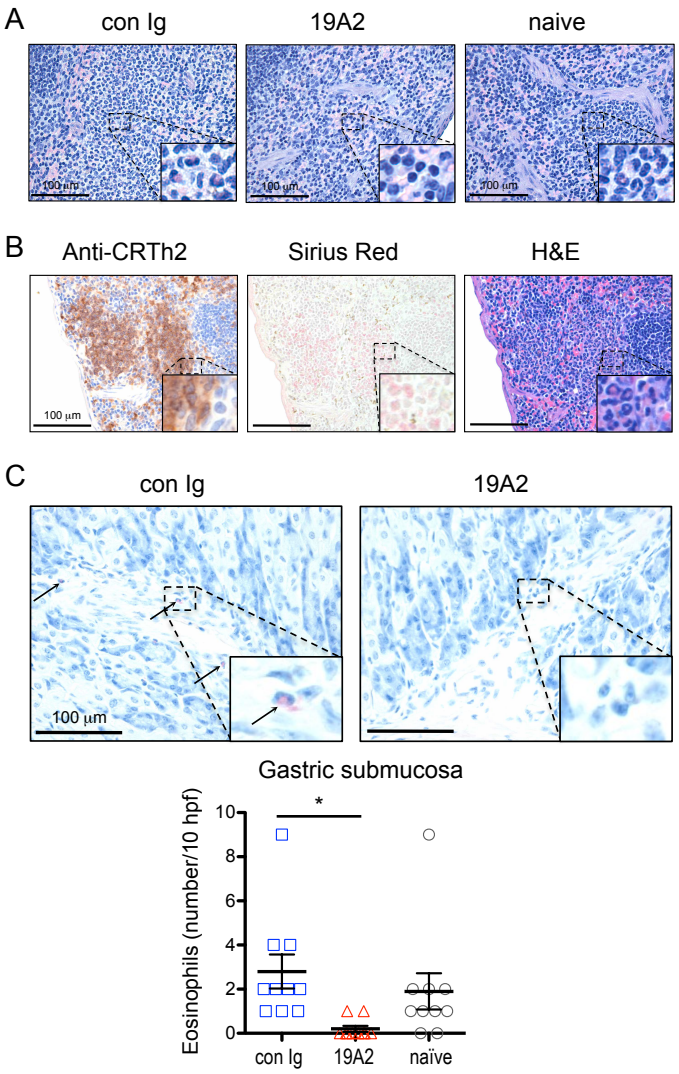
Supplementary Figure 2

Human CRTh2-reactive 19A2 and 8B1 mAbs bind human CRTh2 expressed on 300.19 cells. Reactivity by flow cytometry of mouse anti-CRTh2 antibodies 19A2 and 8B1 (mIgG2a) with 300.19 cells expressing amino-terminal flag-tagged hCRTh2 (upper panel) and with wild-type 300.19 cells (lower panel). Anti-CRTh2 mAb (black line) and isotype control mAb (tinted histogram) were used at 1μg/ml. The anti-Flag mAb (black line) and its isotype control mAb (tinted histogram) were used at 0.7μg/ml. A representative experiment of at least 3 independent experiments is shown.



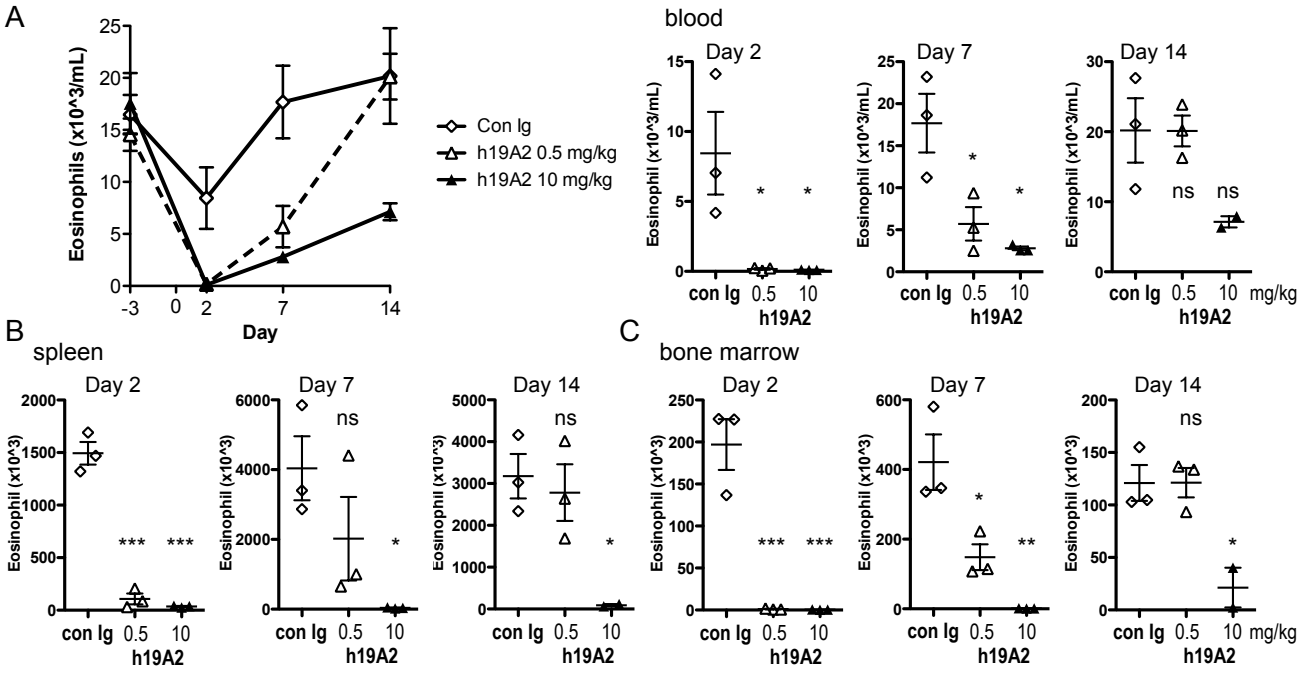
Supplementary Figure 3

Anti-CRTh2 19A2 mAbs deplete basophils and eosinophils in vivo in hCRTh2.BAC.Tg mice. Human CRTh2.BAC.Tg mice were treated i.v. with 100 µg/ mouse control Ig or with anti-hCRTh2 mAb 19A2.mIgG2a.af at 100 µg/mouse (~5mg/kg) or 20 µg/mouse (~ 1mg/kg). The presence of basophils (CD123+FcεRI+) (**A**) and eosinophils (CCR3+) (**B**) was determined by flow cytometry in blood via eye bleed 7 days before dosing and on day 3 as well as on day 7 when mice were euthanized. Spleen and bone marrow cell composition was determined on day 7. Results are mean ± SEM; number of mice per group were n=4 (19A2) and n=3 (control Ig). *, # $p \leq 0.05$, **, ## $p \leq 0.005$, *** $p \leq 0.0005$ (Dunnett's test); *, 100 µg/mouse compared to control Ig; #, 20 µg/mouse compared to control Ig at day 3 and day 7 in blood.



Supplementary Figure 4

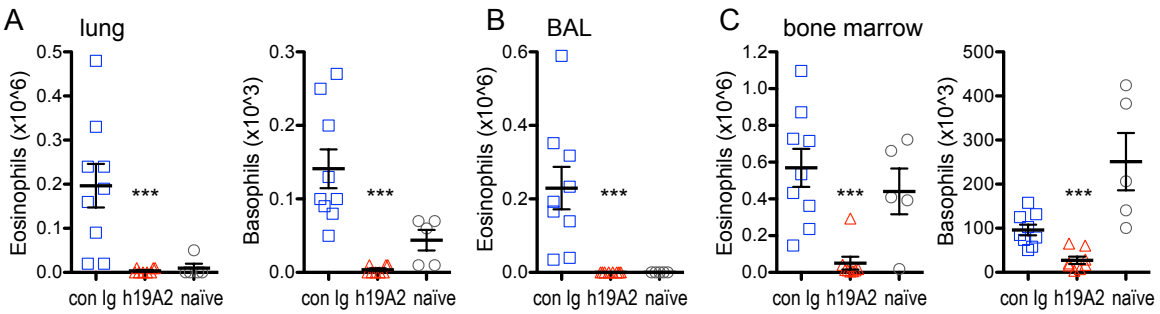
Histological evaluation of eosinophils in spleen and stomach of hCRTh2.BAC.Tg mice after depletion with hCRTh2-specific mAbs. (**A**) Representative images of eosinophil-rich nodules in splenic red pulp evaluated histologically by hematoxylin and eosin staining for data presented in Figure 6E. (**B**) Serial sections of spleen showing that eosinophils in the nodules are anti-hCRTH2 immunoreactive (clone 81.12.4) and readily morphologically recognized by either sirius red or H&E staining as granulocytes with characteristic large eosinophilic cytoplasmic granules and semi-segmented ring-like nuclei. (**C**) Representative images and enumeration of eosinophils in stomach, evaluated histologically by sirius red staining. Results are mean ± SEM. * $p < 0.05$ (two-tailed Student's t-test).



Supplementary Figure 5

Humanized h19A2 mAbs deplete eosinophils in vivo in hCRTh2.BAC.Tg mice.

Human CRTh2.BAC.Tg mice were treated i.v. with 10 mg/kg control Ig or anti-hCRTh2 mAb h19A2.hIgG1 at 10mg/kg or 0.5mg/kg. The presence of eosinophils was determined by flow cytometry, in groups of mice 2, 7 or 14 days after dosing, in blood (**A**), spleen (**B**) or bone marrow (**C**) after euthanization of mice. Three days prior to dosing eosinophil numbers were assessed by flow cytometry in blood that had been collected via eye bleeds. Results are mean ± SEM; number of mice per group were n=3. . * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ (Dunnett's test).



Supplementary Figure 6

Therapeutic treatment with humanized hCRTh2-specific h19A2 clinical candidate depletes basophils and eosinophils from lung in allergic asthma. Human CRTh2.BAC.Tg mice were sensitized with TNP-OVA/alum on day 0 and challenged with 7 daily aerosol administrations of TNP-OVA, starting on day 35 until day 41. Mice were treated with 2mg/kg of a reverse chimera of humanized hCRTh2-specific h19A2.mIgG2a.rc or control mIgG2a antibody on day 38 and day 40. (**A, C**) Eosinophil and basophil numbers as indicated were determined by flow cytometry in lung (**A**) and bone marrow (**C**). (**B**) Eosinophil numbers were assessed in BAL by differential cell count. Results are mean ± SEM; number of mice per group were n=9 (control Ig), n=8 (h19A2) or n=5 (naïve). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ (Dunnett's test).