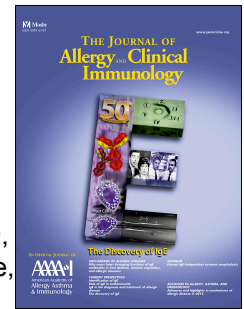


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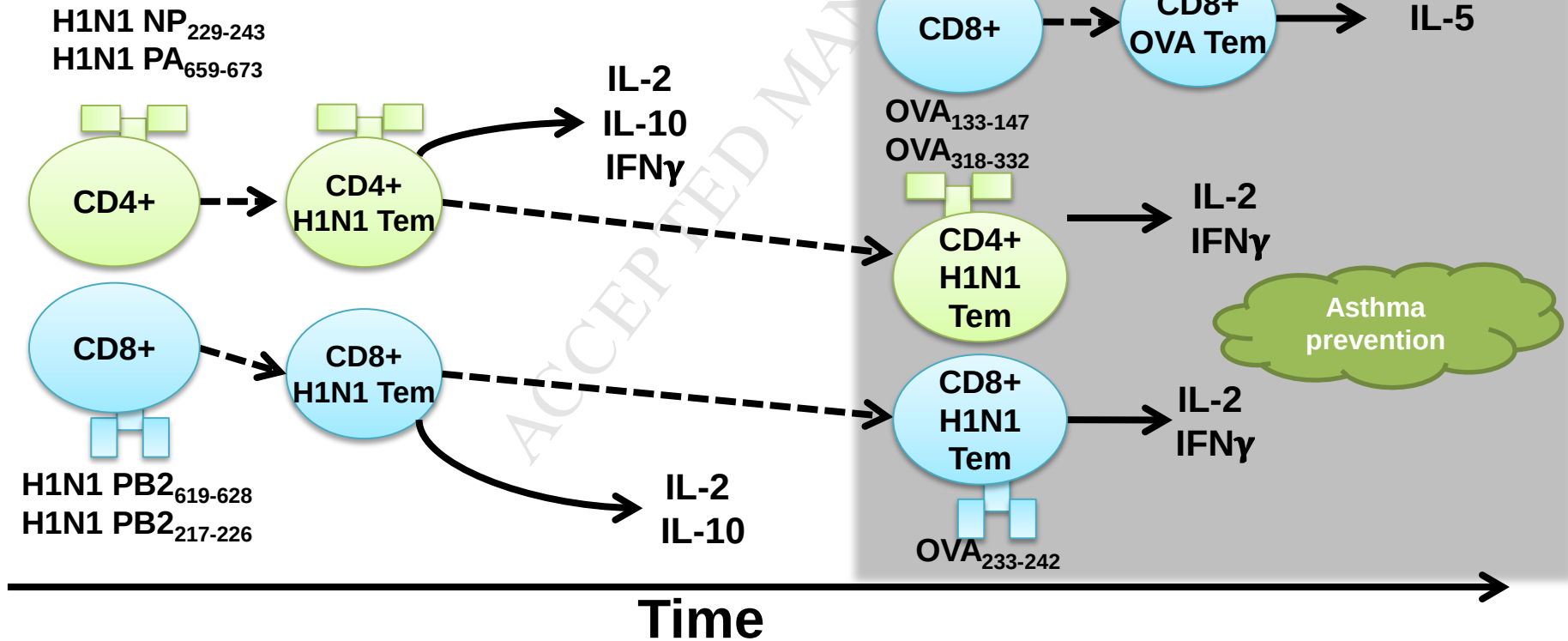
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Virus Infection

Allergen exposure



Influenza-derived peptides cross-react with allergens and provide asthma protection

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COMPETING FINANCIAL INTERESTS

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4.773 words (without title page, list of abbreviations, abstract, figure legends, and list of references), 5 figures, 6 supplementary figures, 1 supplementary table

38 LIST OF ABBREVIATIONS

39	AHR	airway hyperreactivity
40	BAL	bronchoalveolar lavage
41	BLAST	basic local alignment search tool
42	CD	cluster of differentiation
43	CMV	cytomegalovirus
44	Der f	Dermatophagoides farinae
45	Der p	Dermatophagoides pteronyssinus
46	Dpi	days post-infection
47	ELISPOTs	enzyme linked immunospot assay
48	HA	hemagglutinin
49	H1N1	hemagglutinin 1 neuraminidase 1
50	HDM	house dust mite
51	HIV	human immunodeficiency virus
52	HSV	herpes simplex virus
53	IAd, IEd	H2 Class II alleles
54	IEDB	immune epitope database
55	IFN	interferon
56	IFV	influenza virus

57	IL-2, -5	interleukin-2, -5
58	i.n.	intranasally
59	i.p.	intraperitoneally
60	M1, M2	matrix protein 1, 2
61	MDCK	Madin–Darby canine kidney
62	MHC	major histocompatibility complex
63	NCBI	national center for biotechnology information
64	NA	neuraminidase
65	NP	nucleoprotein
66	NS1, NS2	non-structural protein 1, 2
67	OVA	ovalbumin
68	PA	polymerase acidic protein
69	PAS	periodic acid/Schiff
70	PBS	phosphate buffered saline
71	PB 1, 2	polymerase basic protein 1, 2
72	p.f.u.	plaque-forming units
73	POD	peroxidase
74	RNA	ribonucleic acid
75	s.c.	subcutaneous

76 SEM standard error of the mean

77 TCRs T cell receptors

78 Th2 T helper 2

79 Tem T effector memory cell

80

ABSTRACT

Background: The hygiene hypothesis is the leading concept to explain the current asthma epidemic, which is built on the observation that a lack of bacterial contact early in life induces allergic Th2 immune responses. **Objective:** Since little is known about the contribution of respiratory viruses in this context, we evaluated the effect of prior influenza-infection on the development of allergic asthma. **Methods:** Mice were infected with influenza and once recovered, subjected to an ovalbumin or house dust mite-induced experimental asthma protocol. Influenza-polarized T effector memory cells (Tem) were adoptively transferred to allergen sensitized animals prior to allergen challenge. A comprehensive *in silico* analysis assessed homologies between virus- and allergen- derived proteins. Influenza-polarized Tem cells were stimulated *ex vivo* with candidate peptides. Mice were immunized with a pool of virus-derived T-cell epitopes. **Results:** We found in two murine models a long-lasting preventive effect against experimental asthma features. Protection could be attributed about equally to CD4⁺ and CD8⁺ T-effector memory (Tem) cells from influenza-infected mice. An *in silico* bioinformatic analysis identified four influenza and three allergen-derived MHC-class I and –class II candidate T-cell epitopes with potential antigen-specific cross-reactivity between influenza and allergens. Lymphocytes from influenza-infected mice produced IFN γ and IL-2 but not IL-5 upon stimulation with the aforementioned peptides. Immunization with a mixture of the influenza-peptides conferred asthma protection, and peptide immunized mice transferred protection via CD4⁺ and CD8⁺ Tem cells. **Conclusion:** Our results illustrate for the first time heterologous immunity of virus-infected animals towards allergens. This finding extends the original hygiene hypothesis.

105

106 **Key Messages**

- 107 • A preceding influenza virus infection confers long lasting protection from allergen-
108 induced experimental asthma.
- 109 • Protection is attributed to T effector memory cell antigen-specific cross-reactivity.
- 110 • Our results illustrate for the first time heterologous immunity of virus-infected subjects
111 towards allergens with important implications for public health policies regarding
112 vaccination and future strategies for peptide vaccine development.

113

114 **Capsule Summary**

115 Influenza virus infections confer heterologous immunity towards allergens and protect from
116 asthma. T cell cross-reactivity provides a new explanation for the hygiene hypothesis, which
117 is important for public health policies supporting antiviral vaccination.

118

119

120

121 **Keywords:** influenza virus, asthma, hygiene hypothesis, effector memory cells, heterologous
122 immunity

123

INTRODUCTION

More than one billion people worldwide suffer from an allergic disorder, while the prevalence is expected to reach up to 4 billion in 2050s¹. Asthma, a disease characterized by airway hyperreactivity (AHR) and chronic inflammation, constitutes a major part of the epidemic with approximately 300 million afflicted individuals of all ages. The ongoing increase in its prevalence has been associated with profound changes in lifestyle and environmental conditions including sanitation, thus leading to the hygiene hypothesis². Indeed, several investigators^{3,4} observed an inverse relationship between the incidence of infectious diseases and asthma, particularly during the second half of the last century. Although most studies have focused on the protective role of bacteria⁵ and their products, exposure to non-respiratory⁶ and respiratory⁷ viruses has also been inversely associated with atopy and asthma development later in life. To date, functional studies⁸ extending the concept of the hygiene hypothesis to respiratory virus contribution are still lacking. Moreover, severe virus-mediated respiratory tract wheezing illnesses during early life have been linked to the induction of asthma development later in childhood⁹.

A potentially protective role of respiratory viruses from allergy may have important clinical implications; furthermore, IFV is a leading airway pathogen contributing significantly to pulmonary disease burden. We thus aimed to investigate the effects of a preceding IFV infection on the development of allergic airway inflammation. Cellular immunity is largely responsible for heterologous responses to IFV¹⁰⁻¹² and virus-specific CD4⁺ T-effector cells were shown to directly correlate to disease protection among experimentally infected subjects with IFV¹³. Hence we hypothesized that T-cell cross reactivity between IFV and certain environmental allergens may be responsible for any effects over allergic airway inflammation.

METHODS

Mice. Adult 4-6 week-old inbred female Balb/c mice were purchased from Harlan-Winkelmann GmbH (Borchen, Germany).

Virus propagation and titration. The swine-origin pandemic influenza virus A/Hamburg/5/2009 (H1N1) was propagated in Madin–Darby canine kidney (MDCK) (ATCC CCL-34) cells¹⁴ (ovalbumin-free approach). Virus-containing supernatants were collected 2-3 days post infection (dpi) after visible cytopathic effect. The virus was clarified from cellular debris by low-speed centrifugation and infectivity was determined by plaque assay in MDCK cells as described before¹⁵ and expressed in plaque-forming units (p.f.u.) per ml. Aliquots of viral suspension were stored at -80°C until use. For unspecific ex-vivo stimulation and the immunosorbent assay, virus stocks were inactivated by addition of NaHCO₃ (to 0.15%) and beta-propiolactone (Sigma) (1:2000) for 72h at 4°C followed by storage at -80°C.

Influenza infection mouse model. Mice were lightly anesthetized by i.p. injection of Ketamine–Rompun as described above and infected intranasally (i.n.) with 200.000 p.f.u. of H1N1 in 40 µl sterile PBS. Weight loss and survival of infected mice were monitored over a 12-day period (Fig E1a). Animals showing more than 20% of body weight loss were euthanized and documented as dead. Twelve dpi, respective mice were subjected to the OVA-induced or the house dust mite-induced acute asthma model as described below. Additionally, an extended H1N1 infection model was assessed with onset of the OVA-induced asthma model 33 dpi.

Murine ovalbumin (OVA)-induced acute asthma model (Fig E3A). Mice were sensitized to OVA by subcutaneous (s.c.) injection of 10 µg OVA (grade VI; Sigma) without adjuvant in a total volume of 200 µL PBS on days 0, 7 and 14. Subcutaneous injection was performed at

the scruff of the neck and verified by the observation of a fluid bubble forming under the skin¹⁶. The 'Sensitization' phase was followed by 20 min 1% OVA or PBS aerosol treatments on days 24, 25, 26 ('Challenge'). Forty-eight hours after the last aerosol challenge, mice were deeply anesthetized by intraperitoneal injection (i.p.) of 300 mg/kg ketamine hydrochloride (Ketamin, Inresa Arzneimittel GmbH, Freiburg, Germany) plus 30 mg/kg xylazine (Rompun, Bayer Health Care, Leverkusen, Germany) prior to exsanguination by cutting the abdominal aorta.

Murine peptide immunization model. Peptides were synthesized by ProImmune (Oxford, UK) and purity was >90% as determined by HPLC. Animals were immunized i.p with a pool of predicted H1N1 derived MHC-Class I and II T-cell epitopes (PB2₆₁₉₋₆₂₈; PB2₂₁₇₋₂₂₆; NP₂₂₉₋₂₄₃; PA₆₅₉₋₆₇₃) using 100µg/shot emulsified in 1.5mg alum (Pierce, Rockford, IL, USA) on days 0, 6 and 12. On day 17, mice were subjected to our standard OVA-induced asthma model as described above.

Murine house dust mite (HDM)-induced asthma model. HDM allergen whole body extracts (Greer Laboratories, Lenoir, NC), derived from the common European HDM species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* were used to induce allergic airway inflammation as described previously^{17,18}. Briefly, mice were inoculated i.n. with 50 µg HDM on days 0, 7, 14 and 21, while control mice received PBS i.n. on the same days. Inoculum volume was 20 µl for every HDM and PBS exposure and inoculation procedures were performed during light anesthesia as described above. The experiment ended 48h after the last sensitization (on day 23) with euthanizing the mice and subsequent collection and processing of samples as described for the OVA model above.

Flow cytometry. Single cell suspensions were achieved by pushing the respective organs through a 100µm cell strainer (Fisher scientific, Pittsburgh, PA). One-3×10⁵ cells from the

respective organ were washed in cell buffer (PBS^{-/-}, 7.4% EDTA, 5% FCS, pH 7.2) after erythrocyte lysis, incubated with the indicated set of antibodies for 45 min at 4°C, and flow cytometric analysis was performed using a FACS-Canto II flow cytometer (Becton-Dickinson Biosciences, Heidelberg, Germany) and a FACS Diva Software (Becton-Dickinson Biosciences Version 8.0.1). Where indicated, virus-specific CD8⁺ T-cells were labeled with Influenza-A HA₅₃₃₋₅₄₁ H-2Kd-PE labeled tetramer (Prolimmune, Oxford, UK). Fluorochrome-conjugated mAbs to CD3 (V500A2), CD4 (APC GK1.5), CD8 (APC/Cy7 53-6.7), CD45 (PerCP 30-F11), CD44 (FITC IM7), CD62L (PerCP MEL-14), CD69 (PE H1.2 F3), CD103 (Pacific blue 2E7) were purchased from BioLegend, eBioscience, or BD Pharmingen. Effector memory T cells (Tem) cells were defined by CD44⁺ CD62L⁻, naive T cells via CD44⁻ CD62L⁺ and activated T cells by CD69⁺ surface marker expression.

Cell sorting and adoptive cell transfer (Fig E3B). Donor mice underwent H1N1 or PBS treatment respectively. Thirty-three dpi, mice were sacrificed, spleen tissue was homogenized and cells were sorted by flow cytometry with an Aria III (Becton-Dickinson Biosciences, Heidelberg, Germany) using the aforementioned antibody combinations for T-effector memory cell populations (reported purity 99%). Results were analyzed with the DIVA software. Recipient mice received either a single dose of 3x10⁵ CD4⁺/CD8⁺ (CD3⁺ CD44⁺ CD62L⁻) or 3x10⁵ CD4⁺ or CD8⁺ cells (CD8⁺ CD44⁺ CD62L⁻ or CD4⁺ CD44⁺ CD64L⁻) in 100µl sterile PBS into the tail vein 24 h prior to the first OVA-challenge. Control mice (PBS, OVA) were treated according to the OVA-induced asthma model protocol in parallel.

Bronchoalveolar lavage (BAL) acquisition and cytology. By means of a tracheal cannula, BAL was performed with 1 ml PBS containing a protease inhibitor cocktail (Roche, Basel, Germany). An automated CasyTT cell counter (Schäfer Systems, Reutlingen, Germany) was used to determine total leukocyte cell counts and cell-free supernatants were stored at -20°C for cytokine quantification. For differential cell counts, cytospins were prepared, fixed,

and stained with Diff-Quick (Merz & Dade AG, Duding, Switzerland). Standard microscopical and morphological criteria were used to identify cell types (Olympus BH2, Hamburg, Germany; Assistant 345 counter AC-8, Hecht-Assistent, Sondheim, Germany)¹⁶.

Lung histology. Directly after performing the BAL, lungs were fixed with 10% formalin via the trachea, removed and stored. Fixed lung tissue was paraffin embedded and 3µm thick sections were stained with periodic acid/Schiff (PAS). PAS-stained sections were microscopically assessed and random images collected under the 10 × objective¹⁹. The numbers of PAS+ mucus producing goblet cells in the bronchial epithelium were recorded and expressed per mm basement membrane. Inflammation score was defined by the average number of inflammatory cells on an external tangent line between two neighboring airway and blood vessel sections by two investigators blinded to the experimental design.

Cytometric bead array. To quantify cytokines in the BAL, 50 µl of cell free undiluted supernatant from each sample was analyzed using mouse cytometric bead array flex sets (M60-009RDPD, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Individual standards were reconstituted and serially diluted in a 96-well plate and all samples assessed by means of specific antibody-coated 7.5-mm capture beads and phycoerythrin-conjugated detection antibodies²⁰. Cytokine measurements were performed using the BioPlex-200 (Bio-Rad). The standard curve and respective cytokine concentrations were calculated with the BioPlex Manager Software 6.1 (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA). Serum anti-H1N1 IgG titers were measured by ELISA. Flat bottomed 96 well plates (Thermo Scientific Nunc, Roskilde, Denmark) were coated over night with inactivated H1N1 (as described above, 2.5µg/ml) in NaHCO₃ (pH 8.4). Plates were blocked for 2 h with PBS/0.01% Tween-80 (PBST) containing 10% horse serum (blocking buffer) at room temperature. Subsequently, serum samples were

added at a dilution of 1:50 and incubated overnight. After three PBST washes, wells were probed with goat anti-mouse–HRP (Dako Cytomation, Cambridgeshire, UK) (1:2000) for 2 h at room temperature. Plates were washed six times with PBST and signal was developed with BM blue POD substrate (Roche, Risch-Rotkreuz, Switzerland). Reactions were stopped with 2 N sulfuric acid and absorbance was read at 450 nm.

Airway resistance (AR). Lung resistance was measured 24h after the last OVA challenge of our standard OVA-induced asthma model protocol in response to increasing doses of methacholine (2,5-50 mg/ml)²¹. This was performed in tracheostomized anesthetized mice using the flexivent animal mechanical ventilator system (Scireq, Montreal, Canada). Mechanical ventilation was set at 150 breaths/minute with a tidal volume of 0.16 mL/kg and a positive end-expiratory pressure of 3 cm H₂O.

In silico prediction of H-2 Class I & II T cell epitopes and identification of potentially cross-reactive H1N1, OVA and HDM peptides. Amino acid sequences for the A/Hamburg/5/2009 proteins neuraminidase (NA), hemagglutinin (HA), matrix protein 1 (M1), M2, polymerase basic 1 (PB1), PB2, nucleoprotein (NP), polymerase acidic (PA), non-structural 1 (NS1), NS2 as well as for ovalbumin (OVA), *Dermatophagoides pteronyssinus* Der p1, Der p2, Der p 10, and *Dermatophagoides farinae* Der f 1-3, Der f 5-10, Der f 13, Der f 15-16, and Der f 22 proteins were downloaded from the Protein Database of the NCBI. Potential 9- and 10-mer H-2^d Class I (Ld, Kd, Dd alleles) epitopes were identified on NetMHC server 3.4 and those with binding affinity ≤ 500 nM were selected. Virus predicted epitopes were aligned against OVA-derived predicted epitopes and full OVA protein sequence with the use of protein Basic Local Alignment Search Tool (BLAST). Maximum scores and e-values of significant results were screened and best ranking combination of peptides was selected (H1N1 PB2₆₁₉₋₆₂₈, OVA₂₃₃₋₂₄₂, e-value: 4x10⁻⁴). Two additional tools, PRED BALB/c <http://cvc.dfci.harvard.edu/balbc/> and syfpeithi <http://www.syfpeithi.de/index.html> were

used to confirm that best well aligning peptides are predicted as T cell epitopes with strong H-2^d Class affinities. The same procedure was followed for H2 Class II alleles (IAd, IEd) primarily with the use of Immune Epitope Database (IEDB) (selected epitopes demonstrated an affinity score under ≤ 10.00) and predicted affinity of candidate peptides searched in PredBALB/c and syfpeithi tools (best ranking combination being H1N1 NP₂₂₉₋₂₄₃, OVA₁₃₃₋₁₄₇, e-value: 7×10^{-6}). An independent investigator used the aforementioned IEDB tool and among the top 2% scored predicted virus T-cell epitopes (477 Class I 9- and 10-amino acid long, and 812 Class II 15-amino acid long peptides) identified the peptide which contained the minimal number of amino acid mismatches (and maximum number of amino acid matches) with OVA (Class I: H1N1 PB2₂₁₇₋₂₂₆, OVA₂₃₃₋₂₄₂, e-value: 4×10^{-3} ; Class II: H1N1 PA₆₅₉₋₆₇₃, OVA₃₁₈₋₃₃₂, e-value: 2×10^{-3}). Candidate potentially cross-reacting OVA and virus epitopes selected based on both strategies were included in the ex-vivo peptide T cell stimulation and the *in vivo* peptide immunization experiments respectively.

Similar to the first strategy described above, alignments between virus-derived predicted T-cell epitopes, and the entire *D. pteronyssinus* and *D. farinae*-associated allergen sequences (listed above) or the associated predicted Class I and Class II T cell epitopes yielded the following significant combinations of Class II predicted T cell epitopes: H1N1 PB1₃₁₅₋₃₂₉ vs Der f 8₇₀₋₈₄, PB1₃₁₆₋₃₃₀ vs Der f 8₇₀₋₈₄, PB1₃₁₅₋₃₂₉ vs Der f 8₇₁₋₈₅, PB1₃₁₆₋₃₃₀ vs Der f 8₇₁₋₈₅; e-value: 3×10^{-6} for all.

Ex-vivo peptide T cell stimulation. T-cells were isolated from lungs using the Pan T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by FACS sorting of Tem cells as described above. Two $\times 10^5$ cells were incubated in 96 well plates (roundwell) in RPMI 1640 medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Unspecific stimulation experiments were performed by addition of aCD3/aCD28 (0.5µg/ml,

296 2µg/ml), while for virus-specific stimulation 0.6nmol PepTivator® Influenza A NP (defined as
297 'H1N1 Peptide Cocktail', Miltenyi Biotec, Auburn, CA) was added, both for 72h at 37°C. For
298 sequence specific stimulation, spleen cells were incubated with 10µM of OVA₂₃₃₋₂₄₂:
299 LPFASGTMSM, OVA₁₃₃₋₁₄₇: INFQTAADQARELIN, OVA₃₁₈₋₃₃₂: SAESLKISQAVHAAH or
300 unrelated hepatitis B virus (HBV) peptide₂₈₋₃₉: IPQSLDSWWTSL (ProImmune, Oxford, UK).

301 **ELISPOT assay.** After coating 96-well filtration plates (Merck Millipore, Carolina, USA) with
302 anti-mouse IFNγ, anti-IL-5, anti-IL-2 or anti-IL-10 mAbs overnight at 4°C (BD Biosciences,
303 Heidelberg, Germany), 200.000 cells were added and incubated with indicated peptides at
304 37°C in a humidified atmosphere containing 5% CO₂. Cytokine-producing spots were
305 counted automatically using an ELISPOT plate reader (A.EL.VIS, Hannover, Germany), and
306 the median number of spots was determined in triplicate by subtracting the number of spots
307 in the unstimulated wells from those in the peptide-stimulated wells²².

308 **Statistical analysis.** Animal group size was selected based on numbers historically suitable
309 for detecting significant effects in BAL cellularity and cytokines, lung histology and AR.
310 Animals were randomly allocated to experimental groups upon purchase to minimize any
311 bias. There was no investigator blinding unless otherwise stated. Data analysis was
312 performed using the GraphPad Prism (v6.05, La Jolla, CA, USA) software. Exclusion of
313 outlier data points was performed by means of the Grubbs' test (ESD, extreme studentized
314 deviate) in GraphPad. Comparison of variances was performed in GraphPad using the F test
315 and showed similarity between the groups. One-way analysis of variance (ANOVA), followed
316 by the Tukey's multiple-comparison test, was utilized where deemed appropriate. Statistical
317 analysis for H1N1 serology (Fig E1c), AR (Fig. 1e) and ELISPOTs (Fig. 3c) used unpaired
318 two-tailed t-test. $P < 0.05$ was considered statistically significant. Data are shown as
319 mean + SEM. Statistical comparisons are displayed between control groups (PBS/PBS,
320 PBS/OVA), and between PBS/OVA and treated groups, unless stated otherwise.

Study approval.

All animal studies were endorsed by the Federal Authorities for Animal Research of the Regierungspraesidium Giessen, Hessen, Germany, protocols 21/2010, 121/2012, and G48/2016.

RESULTS

Influenza infection mouse model

To investigate the contribution of viral infections to asthma development, a murine model of IFV infection was established. The latter was characterized by moderate to severe airway and lung inflammatory responses, followed by complete recovery and development of long-lasting effector memory cell responses. Animals were infected with a representative pandemic H1N1/2009 influenza A virus, the A/Hamburg/5/2009 strain (referred to as 'H1N1' throughout the manuscript and figures). Airway inflammation peaked approximately on the 10th day post-infection (dpi) along with a drop in body weight, an expected strong influx of primarily neutrophils, macrophages and lymphocytes in the airways, and high levels of TNF α , IFN γ , and IL-6 levels in the bronchoalveolar lavage (BAL) (**Fig E1 and Fig E2**). This acute inflammatory response was followed by a complete recovery and the absence of infectious virus from 12 dpi onwards (**Fig E1B**). The lung immune responses revealed marked increases of both activated CD4⁺ and CD8⁺ T-cells with a reduced CD4⁺/CD8⁺ T-cell ratio due to an enlarged CD8⁺ T cell pool (**Fig E2C**). Importantly, after 20 dpi a persisting CD8⁺ CD44⁺ CD62L⁻ influenza hemagglutinin T-cell epitope-MHC tetramer⁺ memory cell population was present in the lung and was also detected in the spleen (**Fig E2D**).

H1N1 infection and protection from experimental asthma

Following recovery from influenza infection, the animals underwent an experimental asthma protocol using ovalbumin (OVA) as a model allergen. The sensitization phase to OVA lasts roughly three and a half weeks, and this is followed by three OVA aerosol challenges eliciting the asthmatic phenotype. The latter is characterized by the development of eosinophilic airway inflammation with high levels of Th2-cytokines (IL-4, IL-5 and IL-13), mucus production by airway lining goblet cells and increased lung function resistance (**Fig. 1a-e**). When previously H1N1-infected mice were subjected to this protocol, asthma development was largely abrogated, as indicated by a significant prevention of airway inflammation and mucus secretion, influx of eosinophils, reduction of Th2 cytokines, and attenuation of airway hyperresponsiveness (**Fig. 1**). The observed protection was long lasting (**Fig 4e**).

Lung T effector memory-cells in H1N1 infected animals and their effects on allergen-induced asthma.

Due to the endurance of the conferred protection, we hypothesized that T memory cells are responsible for the observed effects and an analysis of respective cell subsets was carried out. Mice protected from experimental asthma (H1N1/OVA) had a higher frequency (**Fig. 2a**) and number (**Fig. 2b**) of lung $CD8^+CD44^+CD62L^-$ T-cells ($CD8^+$ T effector memory, Tem) and similar frequency (**Fig. 2a**) of lung $CD4^+CD44^+CD62L^-$ T-cells ($CD4^+$ Tem) as compared to PBS/OVA animals. Lung $CD4^+CD44^-CD62L^+$ ($CD4^+$ naive T cells) were similar in frequency among the aforementioned experimental groups while $CD8^+CD44^-CD62L^+$ ($CD8^+$ naive T cells) were lower in terms of both frequency and number in the H1N1/OVA versus the PBS/OVA animals (**Fig. 2a, b**). Lung $CD4^+CD69^+$ (activated $CD4^+$) cells were also less in the H1N1/OVA versus the PBS/OVA animals (**Fig. 2b**). Moreover, $CD3^+$ (designated as $CD4^+/CD8^+$) Tem cells from H1N1 infected animals (H1N1 Tem) were sorted and transferred to OVA-sensitized mice. For induction of the inflammatory phenotype, OVA-aerosol

challenges were performed shortly after the transfer as depicted in **Fig E3**. Transfer of H1N1 CD4⁺/CD8⁺ Tem cells significantly prevented airway eosinophilia ($p<0.001$), as well as BAL Th2 cytokines production including IL-4, IL-5 ($p<0.01$), IL-13 as well as IL-6 ($p<0.05$) in these recipients (**Fig. 2c, d and data not shown**). Furthermore, OVA-induced lung inflammation and mucus production was significantly attenuated (**Fig. 2e, f**). In contrast, transfer of equal numbers of Tem cells derived from PBS/control mice had no significant effects (**Fig E4**). Therefore, transfer of H1N1 CD4⁺/CD8⁺ Tem cells almost completely mirrored the effect observed in animals which were previously infected with H1N1, indicating that memory T-cell subsets can transfer protection from experimental asthma. In order to examine the contribution of CD4⁺ and CD8⁺ Tem cells separately, additional transfer experiments were performed (**Fig. 2c-f**). In terms of airway eosinophilia, H1N1 CD4⁺ or CD8⁺ Tem cells individually contributed to only half the protection observed with transfer of the combined H1N1 CD4⁺/CD8⁺ Tem cells (**Fig. 2c**). Transfer of the same cell subsets derived from PBS exposed animals did not confer any protection (**Fig E5**). Other hallmarks of the clinical phenotype such as BAL Th2 cytokine (IL-5) levels were modified by the CD8⁺ Tem subset only (**Fig. 2d**). This pointed to an additive action of H1N1 CD4⁺ and CD8⁺ Tem cells in our model.

Ex-vivo cytokine profiling of H1N1 Tem cells following specific (homologous and heterologous) and non-specific stimulation

The action of CD4⁺ and CD8⁺ H1N1-derived Tem cells was then investigated *in vitro*. As shown in **Figure 3a, b**, anti-CD3/CD28-stimulated H1N1 Tem produced high amounts of IL-10 and IFN γ as compared to cells derived from non-infected animals. When these H1N1 Tem cells were stimulated with a cocktail of recombinant H1N1 peptides containing both

MHC-class I and -class II binding epitopes, CD4⁺ cells excessively produced IL-10 and IFN γ , whereas CD8⁺ Tem cells produced high amounts of IL-10 (**Figure 3a, b**). Next, it was examined how H1N1-derived Tem cells modulate the OVA-induced immune response. For this purpose we searched for possible sequence homology between OVA and viral proteins by extensive *in silico* analysis using databases (NCBI Protein Database), MHC Class I and Class II T-cell epitope prediction tools (NetMHC²³, PRED BALB/c²⁴, syfpeithi²⁵, IEDB²⁶) and alignment tools (BLAST²⁷) as described in detail in “Methods”. Two independent strategies were performed resulting in the identification of candidate peptides with the best amino acid match or BLAST ranking (alignment score and e-value, **Table E1**) that were selected for further experimentation. For OVA, these consisted of one MHC Class I (OVA₂₃₃₋₂₄₂) and two MHC Class II (OVA₁₃₃₋₁₄₇, OVA₃₁₈₋₃₃₂) peptides. For H1N1, two MHC Class I (PB2₆₁₉₋₆₂₈, PB2₂₁₇₋₂₂₆) and two MHC Class II (NP₂₂₉₋₂₄₃, PA₆₅₉₋₆₇₃) predicted T-cell epitopes were selected. Peptide stimulation experiments were performed with ELISPOTS²⁸ for assessment of IL-2, IFN γ , IL-10 and IL-5 producing cells, and spleen cells from virus-infected animals showed a consistent *ex vivo* response to beta-propiolactone-inactivated H1N1 and to the aforementioned H1N1 recombinant peptide cocktail (**Fig. 3c**). They also responded to Class I- (PB2₆₁₉₋₆₂₈, PB2₂₁₇₋₂₂₆) as well as Class II-restricted peptides (NP₂₂₉₋₂₄₃, PA₆₅₉₋₆₇₃) with enhanced IL-2 and IFN γ production (**Fig E6**). Most importantly, cells from virus-infected animals significantly responded not only to OVA protein but also to the identified MHC Class I and Class II OVA-derived peptides with an increase of IL-2 and IFN γ producing cells as compared to stimulation with an unrelated T-cell epitope (HBV₂₈₋₃₉) (**Fig. 3c**). In contrast, numbers of IL-5 and IL-10 producing cells did not increase upon stimulation with the aforementioned OVA peptides (**Fig. 3c**). Conversely, stimulation of T-cells from OVA sensitized mice resulted in a significant increase of IL-2, IL-10, IFN γ , and IL-5 producing cells following stimulation with full OVA protein ($p < 0.001$ for all) and an approximately 2-fold

increase of IFN γ producing cells upon stimulation with inactivated virus. As a control, OVA-derived T-cells responded with increased numbers of IL-5 producing cells when stimulated with MHC Class II predicted T-cell epitope OVA₃₁₈₋₃₃₂ ($p < 0.01$) and with MHC Class I epitope OVA₂₃₃₋₂₄₂. Cells derived from PBS treated/control animals did not significantly react to inactivated virus nor to OVA protein when compared to the HBV₂₈₋₃₉ T-cell epitope (**data not shown**).

Attenuating *in-vivo* effects of H1N1-derived peptide immunization

In order to examine if epitope cross-reactivity between H1N1 and the allergen OVA accounts for the asthma preventive effect, mice were immunized with a mixture of the candidate H1N1-derived Class I (PB2₆₁₉₋₆₂₈, PB2₂₁₇₋₂₂₆) and Class II (NP₂₂₉₋₂₄₃, PA₆₅₉₋₆₇₃) peptides and experimental asthma was triggered thereafter. Peptide immunization alone did not have any impact on BAL cellularity and cytokine levels, as well as on lung naïve and activated T cell counts (**Fig. 4a-d**). In contrast, peptide immunization significantly suppressed OVA-mediated airway eosinophilia by approximately 50%, along with BAL Th2 cytokine production ($p < 0.01$ for IL-5, $p < 0.05$ for IL-13 and trend-wise for IL-4) (**Fig. 4a, b**). **Figure 4e** shows the degree of suppression of OVA-induced BAL total number of cells, eosinophils and IL-5 levels when H1N1 infection occurred 12 or 33 days before the first OVA sensitization, compared to peptide immunization. Whereas intranasal virus inoculation elicits a primary immune response in the lung, mice which were peptide-immunized induced a primary response in the local lymph nodes. Despite the distant site of exposure, a protective effect could be elicited in the lung.

Subsequently, CD4⁺ or CD8⁺ Tem cells from animals immunized with the same aforementioned mixture of the candidate H1N1-derived Class I and Class II peptides were

sorted and transferred to OVA-sensitized mice. The latter were challenged with OVA-aerosol shortly after the transfer as described above. Transfer of CD4⁺ Tem or CD8⁺ Tem cells from peptide immunized mice significantly reduced airway total cell and eosinophil infiltration ($p < 0.05$ and $p < 0.01$ respectively) (**Fig. 4f**).

Impact of a preceding H1N1 infection on HDM-induced asthma

Furthermore, it was investigated whether a preceding influenza infection would also have an asthma protective effect in the clinically more relevant mouse model of house dust mite (HDM) airway sensitization. Indeed, H1N1 infection significantly suppressed HDM-induced airway eosinophilia (**Fig. 4g**) and lung inflammation (**Fig. 4h, i**), and this was accompanied by a significant reduction in mucus production (**Fig. 4h, i**). Similarly to the OVA model, frequencies of lung CD4⁺ Tem cells were similar and of CD8⁺ Tem cells higher in the H1N1/HDM group as compared to the animals that had not been previously infected (**Fig. 4j**). An *in silico* analysis yielded significant similarities (e-value 3×10^{-6}) between virus PB1 protein and Der f 8 predicted Class II T cell epitopes (see Methods for details), which may account for the protective effects observed in conjunction to our HDM model.

DISCUSSION

We have shown a respiratory virus-mediated protection against experimental asthma that is long-lasting. Furthermore, results of our transfer experiments indicate that CD4⁺ and CD8⁺ Tem cells are largely responsible for the protection. These cells, when isolated from virus-infected animals respond to cross-reactive virus- and OVA-related MHC Class I and Class II peptide sequences, and mice immunized with a pool of these virus-derived T-cell epitopes

demonstrated protection from allergen-induced asthma. To our best of knowledge, this is the first evidence of heterologous immunity towards allergens.

Our results highlight for the first time virus-mediated T cell cross-reactive responses as a potential mechanism for asthma protection. Virus-specific T memory cell cross-reactivity has previously been shown to other antigens including alloantigens²⁹, virus-related^{10–13,30–33} and totally unrelated^{34–37} pathogens, as well as autoantigens³⁸. Thus, memory T cells established by seasonal human influenza A infection cross-react with avian influenza A¹¹, cross-reactive memory T cells afford heterosubtypic protection upon H7N9 in humans and animals¹², while surprisingly cytomegalovirus (CMV)-, human immunodeficiency virus (HIV)-1-, and herpes simplex virus (HSV)-specific memory T cells were found in non-infected individuals³⁴. The latter cells exhibited extensive cross-reactivity to peptides derived from other microbial genomes including unrelated bacteria³⁴. T cells expressing autoreactive T cell receptors (TCRs) may occasionally bypass immune system 'safety checks' and inhabit the periphery. If such T cells become activated in response to a virus-derived peptide and subsequently become effector T cells, they may then cross-recognize a self-derived peptide to cause autoimmune reactions³⁹ (molecular mimicry). Several autoimmune disorders have been previously related to influenza infection or vaccination, including Guillain-Barre syndrome^{40,41}, narcolepsy⁴² and rheumatic inflammatory diseases such as giant cell arteritis and polymyalgia rheumatic⁴³.

Our findings now expand this concept of heterologous reactivity to allergens. Indeed, the repertoire of $\alpha\beta$ T cell receptors (TCRs) is overshadowed by the multitude of possible non-self peptide-MHC complexes; thus, our immune system may only manage if every T cell recognizes several peptides and consequently is cross-reactive³⁹. Since memory T cells comprise the largest lymphocyte subpopulation during the major part of the human lifespan, these cells play a central role in the response to infectious and non-infectious antigens over many decades⁴⁴. Therefore, the age-dependent increase in the frequency of memory T cells

inversely correlates with susceptibility to infection⁴⁵. In this context, an increasing pool of memory T cells may play an important role in the protection against T cell-mediated chronic inflammatory disease.

T cells are ample at the airway mucosa and lung tissue, and virus-specific T cells increase in frequency and number during infection^{46–48}. Such cells in principle recognize fairly conserved internal viral proteins and are thus shown to be able to mediate cross-protection against infections with serologically distinct virus strains^{20,49}. They are initially primed with antigen carried to draining lymph nodes by activated dendritic cells⁵⁰. Following TCR binding, T cells differentiate and promptly form an enlarged effector pool. CD8⁺ T cells become cytolytic and detect virus-infected cells by recognizing MHC Class I virus peptides, while CD4⁺ T cells bind to MHC Class II virus peptides and differentiate into a variety of helper T cell subsets, which produce specific cytokine combinations. The protective response to respiratory viruses is characteristically dominated by IFN- γ and is shifted towards a Th1 response leading to viral clearance^{51–53}. Subsequently, effector T cell pools contract and leave long-lived memory T cells behind, ready to fight a secondary infection.

Memory T cells following IFV infection are mainly characterized by Th1 (CD4⁺) and Tc1 (CD8⁺) phenotypes. Cross-reactivity could represent a major mechanism in eliciting memory cell populations, which recognize allergens and produce Th1 cytokines upon recall. In our setting, CD4⁺ and/or CD8⁺ Tem cells from virus-infected or H1N1 peptide-immunized mice confer asthma protection potentially via Th1 cytokine production following stimulation with ovalbumin-derived cross-reactive T cell epitopes (**Fig. 5**). Such a milieu counteracts harmful Th2 responses against allergens and it has previously been shown that strategies accentuating IFN γ -mediated immune responses protect from experimental asthma⁵⁴.

Interestingly, mice protected from experimental asthma (H1N1/OVA) demonstrated higher numbers of BAL macrophages (**Fig. 1a**). The role of these cells and associated innate immune mechanisms in asthma protection effects remains to be determined. Indeed, we

have observed an important role of macrophages in bacterial-mediated protection from experimental asthma (unpublished data), which we are currently investigating. Also of note, is the reduction of lung naïve T cells in mice previously immunized with the H1N1 peptide mix as compared to those which were subjected to the OVA-induced asthma protocol alone (**Fig 4d**). This reduction was not observed in terms of cell frequency (%) (**Fig. 4c**), which suggests that the total number of T cells in the lungs is reduced as a result of compartmentalization associated with prior peptide immunization.

The mechanism described in our work provides a new explanation for the inverse association between prevalence of infectious diseases and asthma, particularly during the second half of the last century^{3,4}. Certainly, decreased exposure to pathogens may have narrowed the spectrum and number of memory T cells, which are available to cross-react to environmental antigens such as allergens. Along these lines, mild repetitive viral infections could potentially exert a beneficial effect as opposed to single severe lower respiratory tract infections, which predispose to wheezing in childhood⁹. It remains to be tested whether this concept may involve also other (RNA) viruses and a broader spectrum of environmental allergens. Additionally, the human virome is known to be highly specific to its individual host^{55,56}, relatively stable⁵⁶, and composed of both eukaryotic viruses and a large number of bacteriophages⁵⁷. It is possible that early in life, an individual's airway virome may contribute to asthma protection via heterologous immune responses highlighted with our work. Our present findings may have important implications for public health policies and advocacies supporting influenza (and other viral) vaccination as a preventive measure against asthma. Indeed, the observation that suitable virus antigenic exposure may prevent asthma development extends the hygiene hypothesis which has primarily attributed the current asthma epidemic to insufficient bacterial exposure. Finally, our findings may aid the design of novel peptide vaccines containing pathogen associated T cell epitopes that could lead to allergen tolerance.

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FIGURE LEGENDS

FIG 1. OVA-induced asthmatic phenotype is attenuated by a preceding H1N1 infection. **A**, Infiltrating cells in the BAL quantified by visual cell quantification. **B**, Cytokine levels in the BAL quantified by a cytokine bead array. **C**, Representative photomicrographs of lung sections for visualization of inflammatory cell infiltration between airways and blood vessels, and goblet cell formation in H1N1/OVA-treated mice compared to PBS/OVA. Magnification

10x. **D**, Quantification of mucus producing goblet cells and inflammation on lung histology sections. **E**, Airway resistance (AR) measurement by increasing administration of nebulized methacholine (MCh). BAL, bronchoalveolar lavage; BV, blood vessel; AW, airway. Data are shown as mean + SEM. Significant comparisons between PBS/PBS to PBS/OVA and PBS/OVA to H1N1/OVA or H1N1/PBS are indicated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. AR (**FIG 1E**) used unpaired two-tailed t-test. Group size n=10-16.

FIG 2. The role of virus-polarized T effector memory-cells in the OVA-induced mouse model. **A**, Lung tissue homogenate was analysed using flow cytometry with T-cell surface markers as indicated. Shown are representative flow cytometry density plots of a single mouse per group. **B**, Quantification of total, naive (CD44⁻ CD62L⁺), activated (CD69⁺) and effector memory (CD44⁺ CD62L⁻) CD4⁺ (upper panel) and CD8⁺ (lower panel) T-cells in lung homogenate. **C-F**, Transfer of 300,000 CD4/8⁺ CD44⁺ CD62L⁻ effector memory T (Tem)-cells from H1N1-infected mice attenuates OVA-induced acute allergic airway inflammation, while transfer of corresponding CD4 Tem cells or CD8 Tem confers only partial protective effects. **C**, Differential BAL cell count after transfer of Tem cells derived from H1N1-treated mice. **D**, Percentage suppression of OVA-induced BAL IL-5 following T effector memory-cell transfer derived from H1N1 exposed animals quantified by a cytokine bead array. **E-F**, Lung sections (representative immunohistochemistry images are shown in **E**) were quantified for mucus producing goblet cells and inflammation (**F**). Data are shown as mean + SEM. Significant comparisons between PBS/PBS to PBS/OVA and PBS/OVA to recipient groups are indicated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Group size n (control)=8, n (donor)=10, n (acceptor)=6-8.

738

739 **FIG 3.** *Ex vivo* stimulation of H1N1 polarized lymphocytes with homologous and
740 heterologous peptides results in an increase of IFN- γ release. Cells were isolated from H1N1
741 or PBS treated mice and restimulated *ex-vivo* as indicated. **A**, FACS sorted CD4⁺ CD44⁺
742 CD62L⁻ or **(B)** CD8⁺ CD44⁺ CD62L⁻ cells from H1N1 or PBS treated mouse lungs were
743 subjected to restimulation with aCD3/CD28 (upper row) or Influenza A (H1N1) NP peptide
744 pool (lower row). **C**, Frequency of IL-2, IFN- γ , IL-5 and IL-10 spot forming cells by ELISpot
745 displayed as fold induction. Data are shown as mean + SEM. Significant comparisons to
746 PBS control group are demonstrated in **(A, B)**. In **(C)** comparisons are depicted against
747 stimulation with unrelated HBV₂₈₋₃₉ peptide. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. One-
748 way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test for **A & B**
749 and unpaired two-tailed t-test in **C**. Group size n=10.

750

751 **FIG 4.** Attenuating *in vivo* effects of H1N1 related immune polarization. **(A-D)** H1N1 derived
752 cross-reactive peptide mix reduces airway eosinophilia. **A**, Cellular composition of the BAL
753 after immunization with peptide mix. **B**, Cytokine levels in the BAL quantified by cytokine
754 bead array. **C**, Representative flow cytometry density plots of T memory cell subpopulations
755 of a single mouse per group in the lung. **D**, Quantification of total, naive (CD44⁻ CD62L⁺),
756 activated (CD69⁺) and effector memory (CD44⁺ CD62L⁻) CD4⁺ (upper panel) and CD8⁺
757 (lower panel) T-cells in lung homogenate. **E**, Fold induction comparisons of inflammatory
758 hallmarks (BAL total, BAL eosinophils, BAL IL-5) compared to OVA in H1N1/OVA, extended
759 H1N1 infection and peptide mix/OVA. Significant differences compared to OVA animals are
760 indicated with asterisks. **F**, Differential cell quantification in the BAL after peptide mix
761 polarized Tem cell transfer. **(G-K)** The HDM - induced asthmatic phenotype is attenuated by
762 a preceding H1N1 infection. **G**, Differential cell quantification in the BAL. **H**, Representative

763 photomicrographs of lung sections (for further description, please, refer to the legend of **FIG**
764 **1C**). **I**, Quantitative analysis of lung histology sections for mucus producing goblet cells and
765 inflammation. **J**, Representative flow cytometry density plots of T memory cell
766 subpopulations in the lung of a single mouse representative for each group. **K**, Quantification
767 of total, naive (CD44⁻ CD62L⁺), activated (CD69⁺) and effector memory (CD44⁺ CD62L⁻)
768 CD4⁺ (upper panel) and CD8⁺ (lower panel) T-cells in lung homogenate. HDM, house dust
769 mite. Data are shown as mean + SEM. Significant comparisons from PBS/PBS to PBS/OVA
770 and PBS/OVA to Peptide mix/OVA or Peptide mix/PBS are indicated (**A**, **B**, **D**, **F**). Significant
771 comparisons from PBS/PBS to PBS/HDM and PBS/HDM to H1N1/HDM or H1N1/PBS are
772 indicated (**G**, **J**, **K**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. One-way analysis of variance
773 (ANOVA) followed by Tukey's multiple-comparison test. Group size n=8.

FIG 5. A preceding influenza virus infection leads to increased number of Th1 cytokine-producing T cells following stimulation with cross-reactive OVA T-cell epitopes. Virus-derived MHC-class I (PB2₆₁₉₋₆₂₈, PB2₂₁₇₋₂₂₆) and class II (NP₂₂₉₋₂₄₃, PA₆₅₉₋₆₇₃) T-cell epitopes prime T effector memory cells (Tem) to a production of IL-2 and IFN- γ upon exposure with cross-reactive OVA-derived MHC-class I (OVA₂₃₃₋₂₄₂) and class II (OVA₁₃₃₋₁₄₇ or OVA₃₁₈₋₃₃₂) T-cell epitopes. Exposure to MHC Class I (OVA₂₃₃₋₂₄₂) or MHC Class II (OVA₁₃₃₋₁₄₇ or OVA₃₁₈₋₃₃₂) OVA epitopes alone leads to accentuated T cell IL-5 production.

Supplementary Figure 1 (Fig E1)

Intranasal (i.n.) infection of Balb/c mice with influenza virus A/Hamburg/05/09 (H1N1) results in mild to moderate weight loss and detection of virus in lung tissue and BAL. **A**, Mice infected i.n. with 200,000 plaque forming units (p.f.u.) of virus were monitored daily for body weight. **B**, Infectious virus particles can be detected up to day 8 in lung homogenate and BAL. **C**, Serum anti H1N1-IgG titers assessed 40 days post infection (dpi), n.d., not detectable. *** $P < 0.001$. Group size n=8

Supplementary Figure 2 (Fig E2)

Fig E2. Infection kinetics peak mainly between 6 and 10 dpi. **A**, Differential BAL cell count. **B**, Cytokine quantification in the BAL. **C**, FACS quantification of total and activated T-cell populations in lung homogenate. **D**, Quantification of CD8⁺ CD44⁺ CD62L⁻ T-cells which bound to H1N1 HA₅₃₃₋₅₄₁ H-2Kd tetramers. For **D**, only comparisons to 0 dpi are shown. ** $P < 0.01$, *** $P < 0.001$. Group size n=6-8

Supplementary Figure 3 (Fig E3)

Fig E3. Experimental protocol for adoptive cell transfer in a murine OVA-induced acute allergic airway inflammation model. Parallel to PBS and OVA control groups (**A**), 33 days following intranasal (i.n.) H1N1 infection or PBS exposure, T effector memory-cells (CD4⁺ / CD8⁺; CD4⁺ or CD8⁺ / CD44⁺ CD62L⁻) were isolated and a total of 300.000 cells injected intravenously (i.v.) (100µl) into the tail vein of OVA sensitized recipient mice. Twenty four hours later, OVA challenge was performed on days 24, 25 and 26 since initial OVA sensitization. Analysis was performed 48h after the last challenge (**B**). s.c., subcutaneous.

Supplementary Figure 4 (Fig E4)

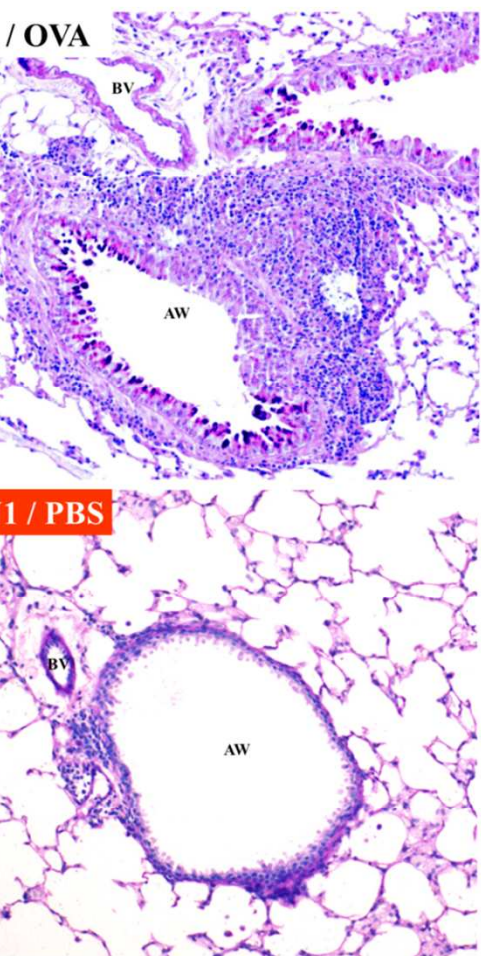
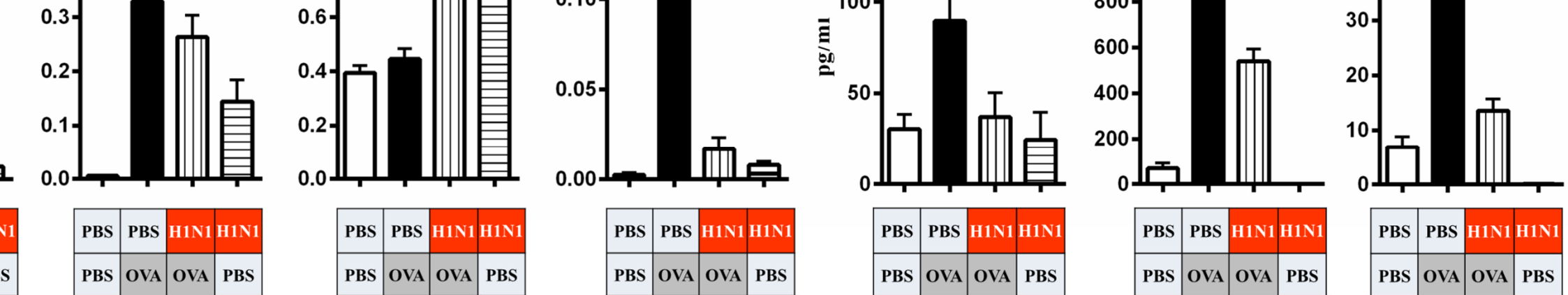
Fig E4. T effector memory-cells derived from PBS exposed animals do not confer protection from experimental asthma. **A**, Percentage suppression of OVA-induced BAL IL-5 following T effector memory-cell transfer (CD4⁺/CD8⁺ CD44⁺ CD62L⁻; CD4⁺ or CD8⁺ CD44⁺ CD62L⁻) derived from PBS exposed animals. **B**, Quantitative evaluation of PAS-stained histology sections. Group size n=8

Supplementary Figure 5 (Fig E5)

Fig E5. T effector memory-cells derived from PBS exposed animals do not alter BAL cell composition shown by differential BAL cell analysis.

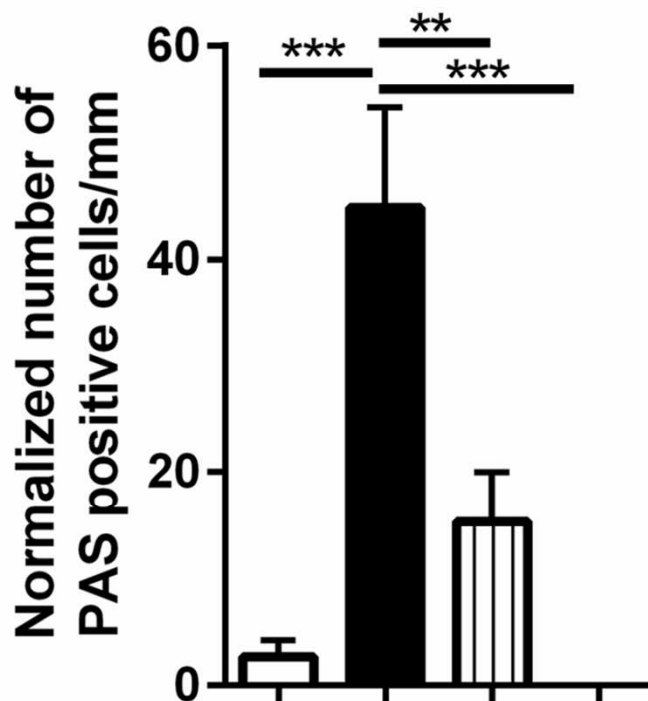
Supplementary Figure 6 (Fig E6)

Fig E6. *Ex-vivo* stimulation of H1N1 polarized lymphocytes with homologous and heterologous peptides results in an increase of IFN γ and IL-2 release. Frequency of IL-2, IFN γ , IL-5 and IL-10 spot forming cells by ELISpot displayed as fold induction. Data are shown as mean +SEM. Significant comparisons are depicted against stimulation with unrelated HBV₂₈₋₃₉ peptide. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Unpaired two-tailed t-test. Group size n=10



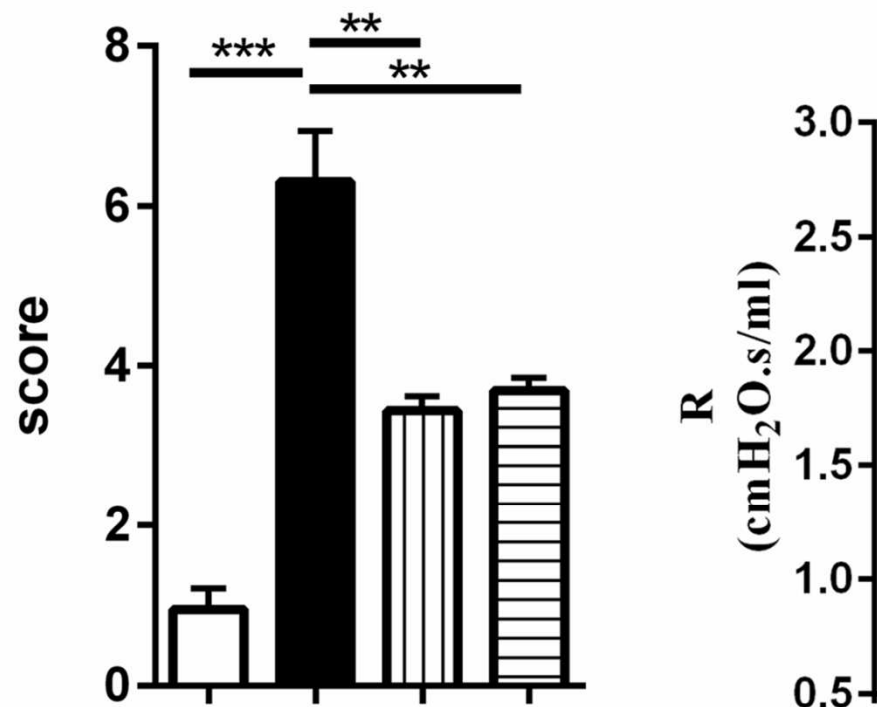
d

Goblet cells



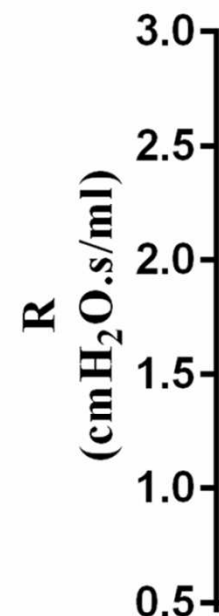
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Challenge	PBS	OVA	OVA	PBS

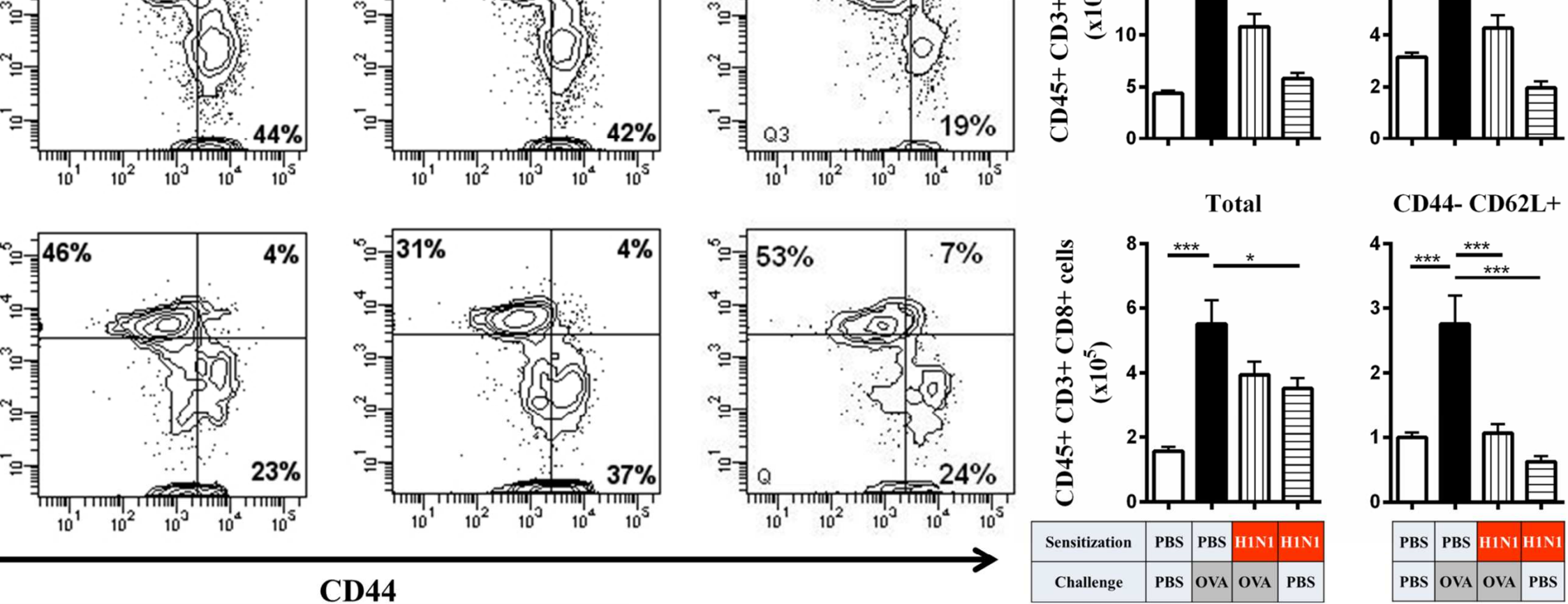
Inflammation



PBS	PBS	H1N1	H1N1
PBS	OVA	OVA	PBS

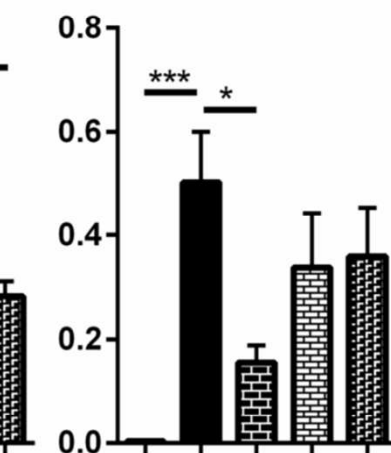
e





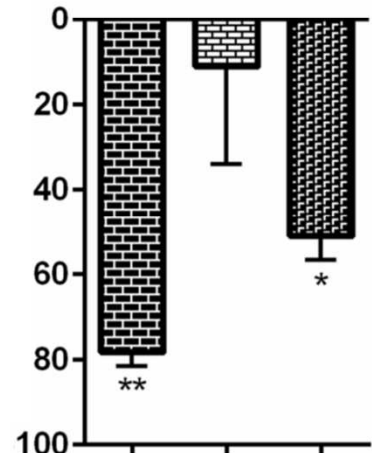
CD44

Lymphocytes



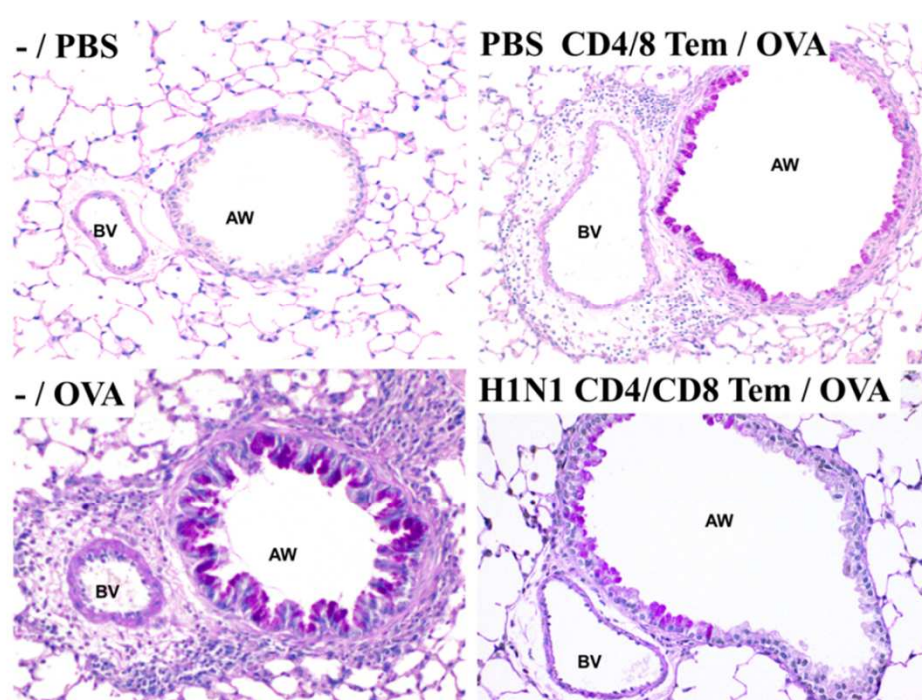
H1N1	D8	tem
-	-	-
-	-	-
H1N1	CD4/8	Tem
H1N1	CD4	Tem
H1N1	CD8	Tem
PBS	OVA	OVA
PBS	OVA	OVA

d

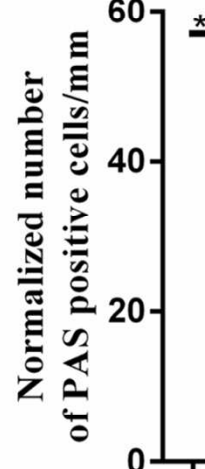


H1N1	CD4/8	TEM
H1N1	CD4	TEM
H1N1	CD8	TEM

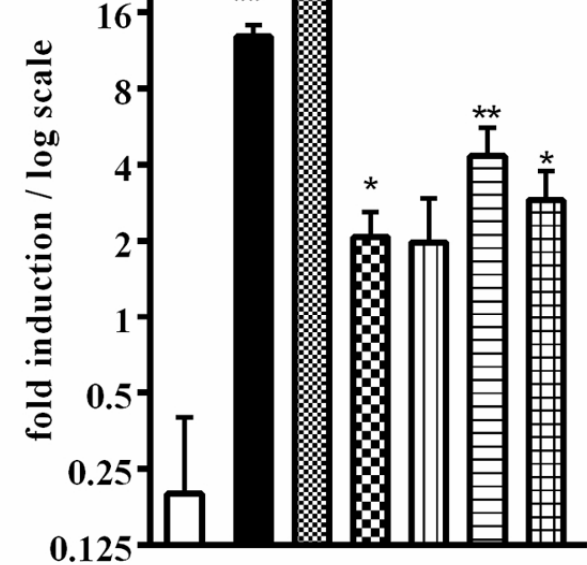
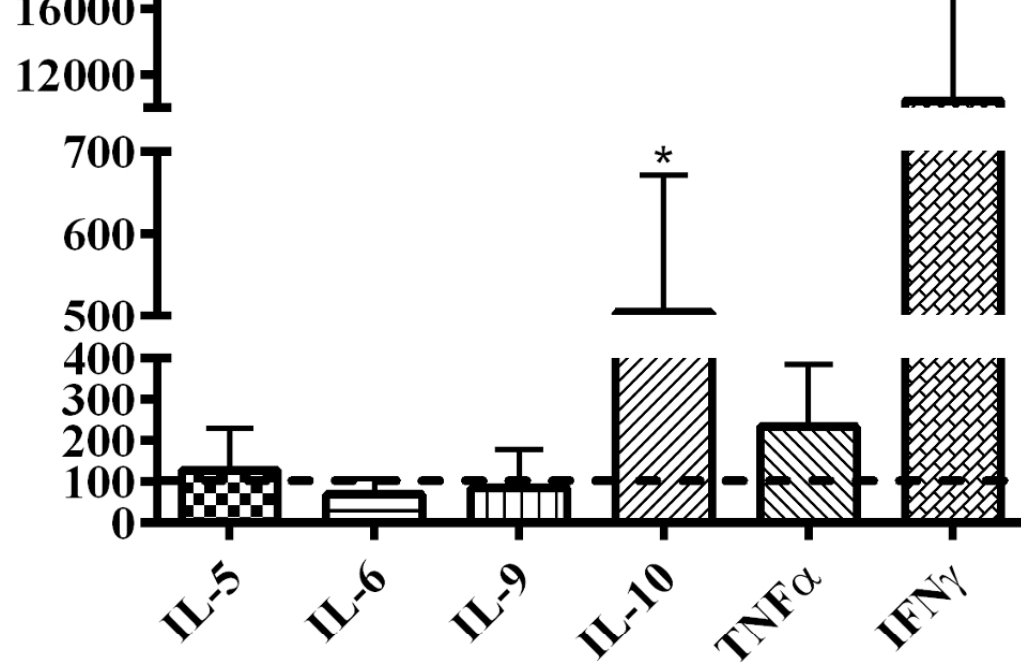
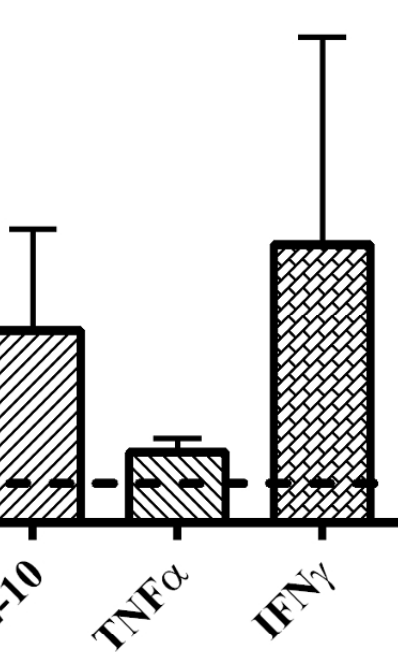
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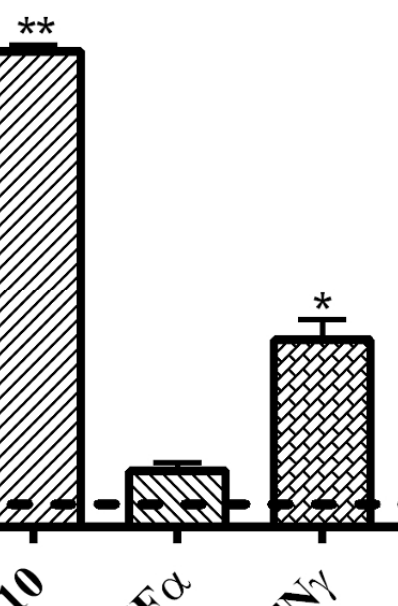
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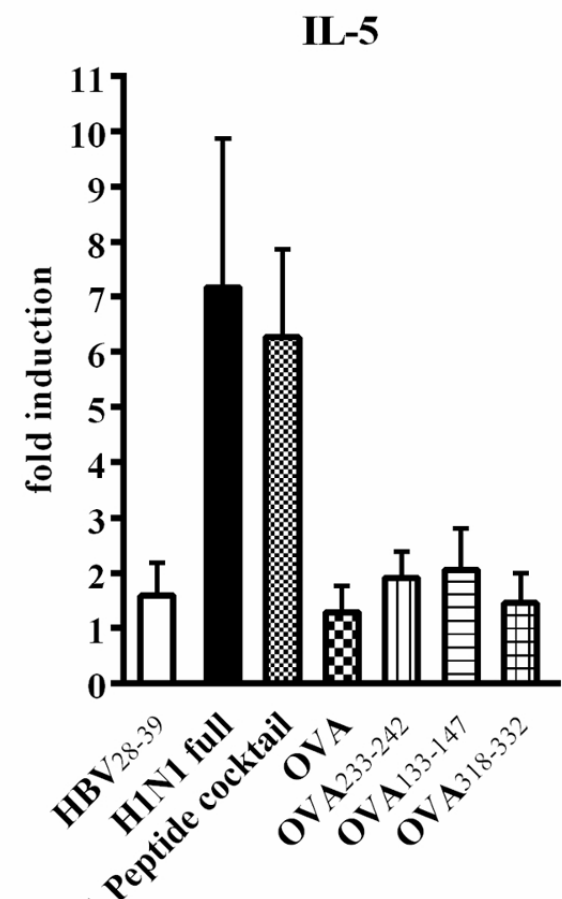
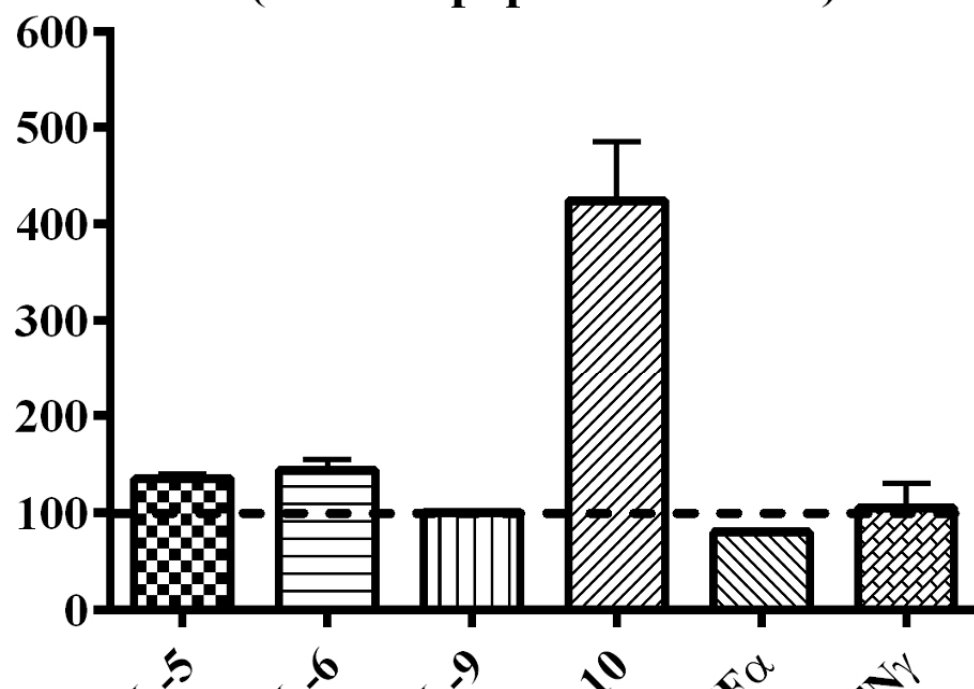
Donor	-
Recipient	PBS

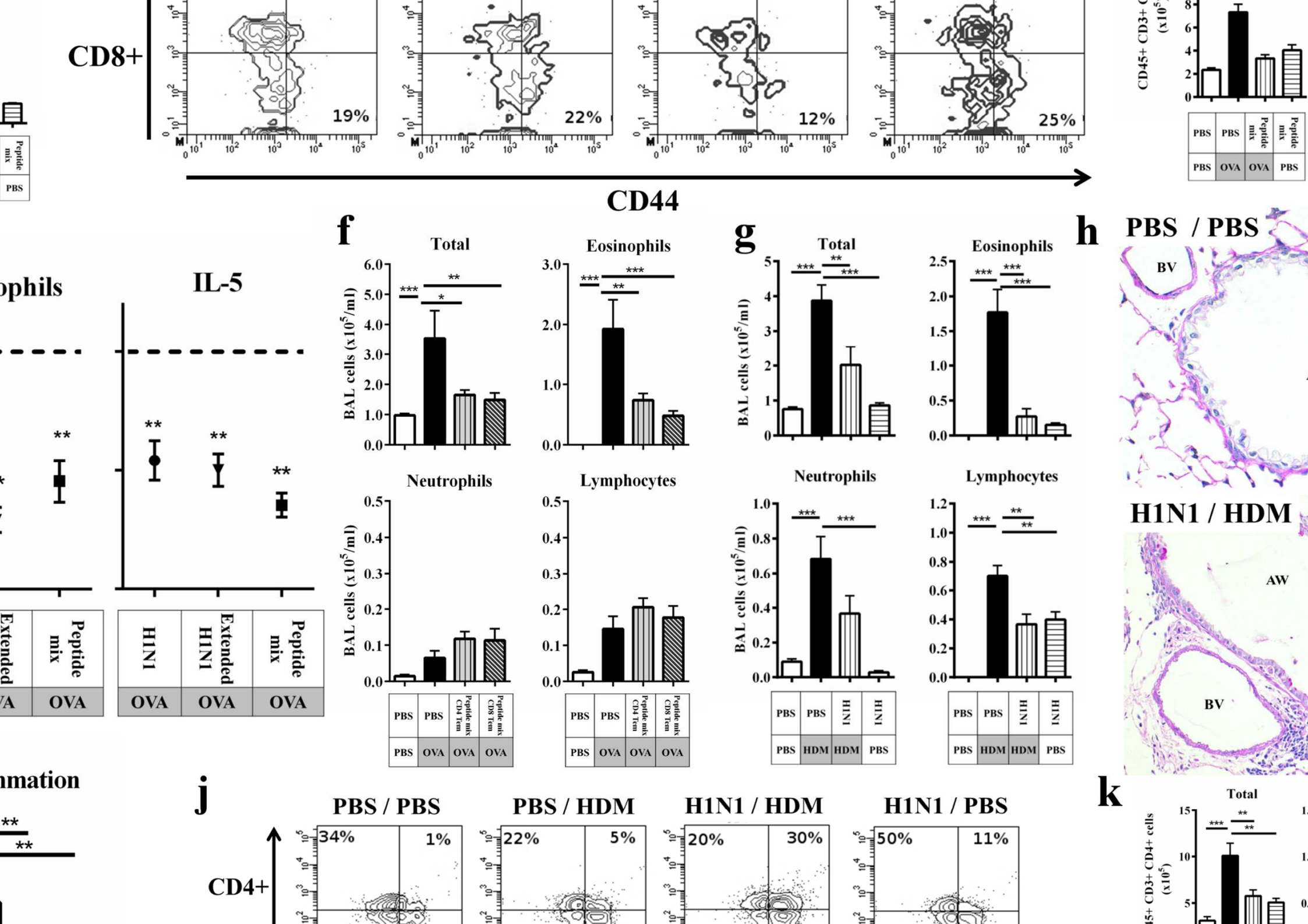


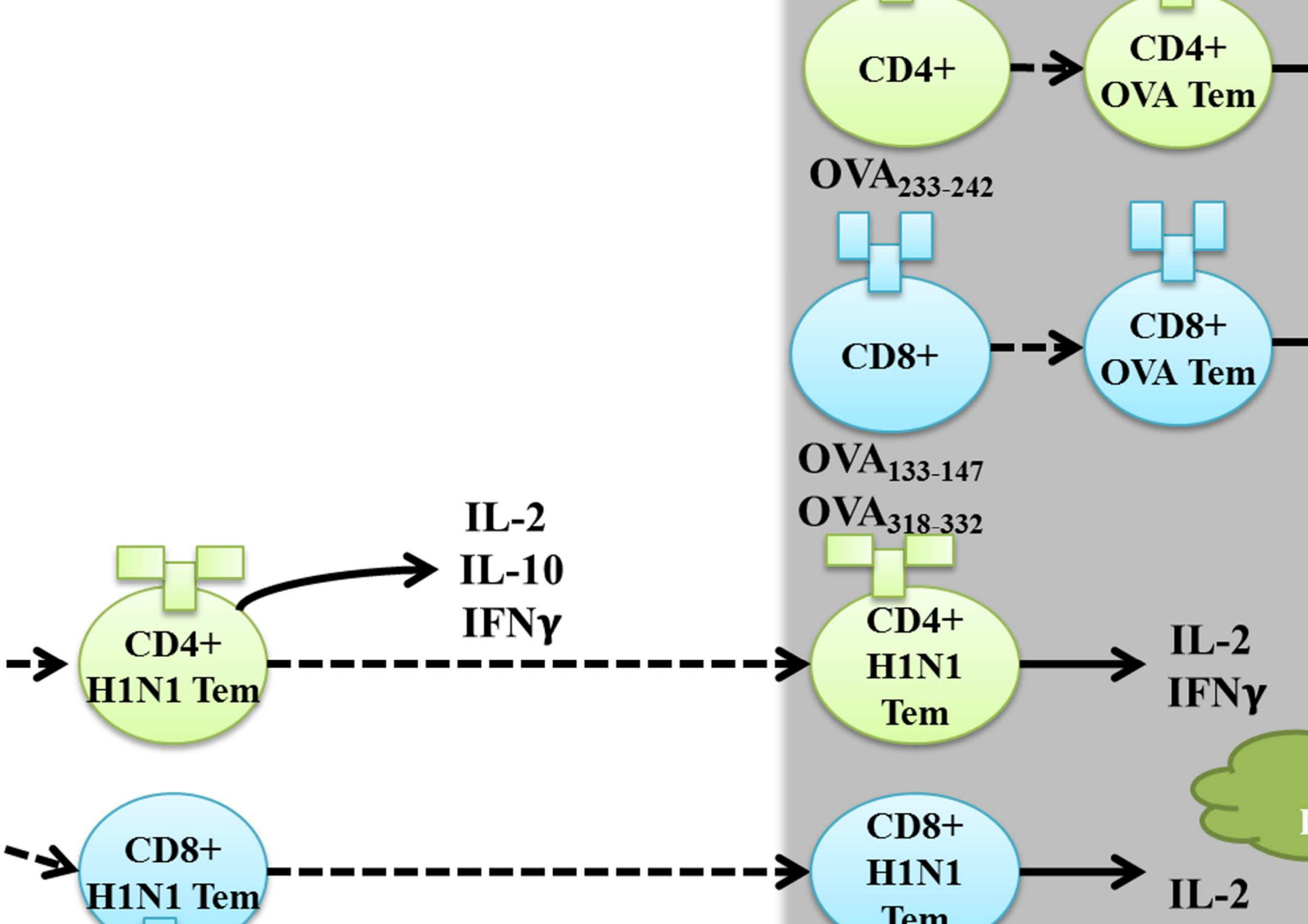
CD8+ CD44+ CD62L-
(+H1N1 peptide cocktail)



CD8+ CD44+ CD62L-
(+H1N1 peptide cocktail)







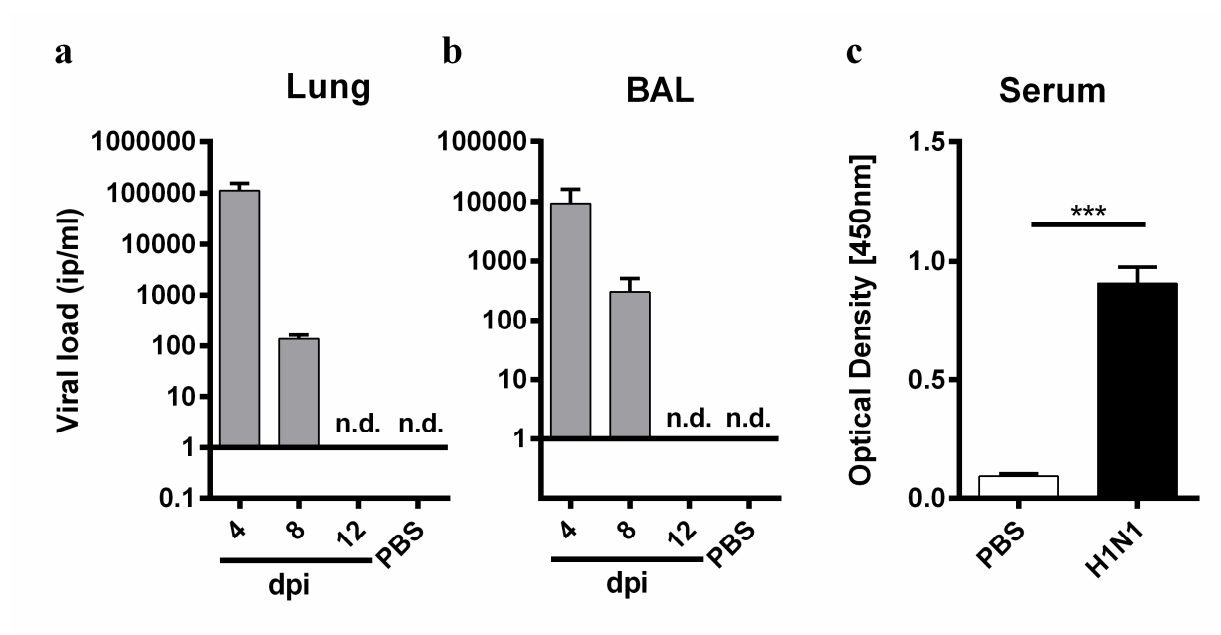


Fig E1.

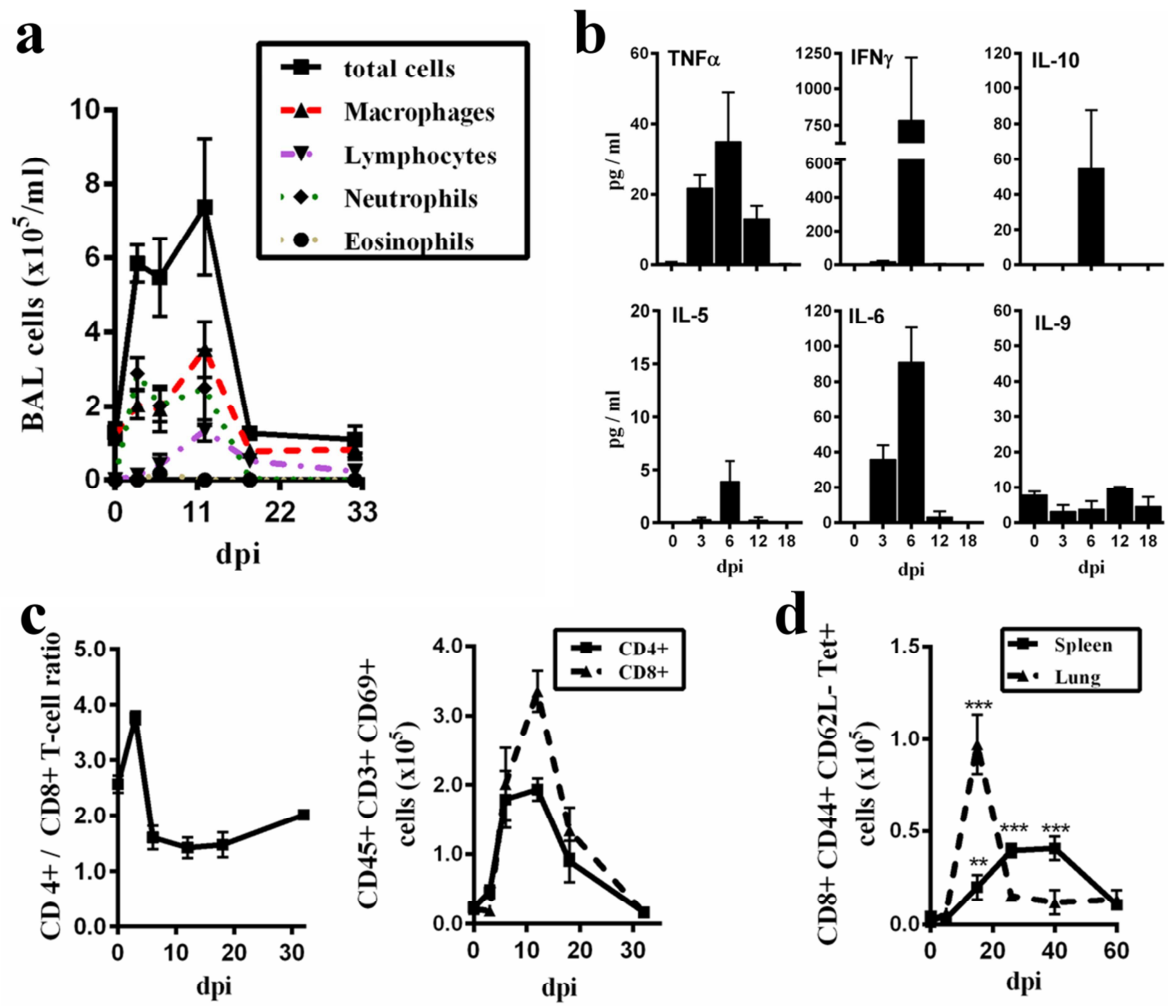


Fig E2.

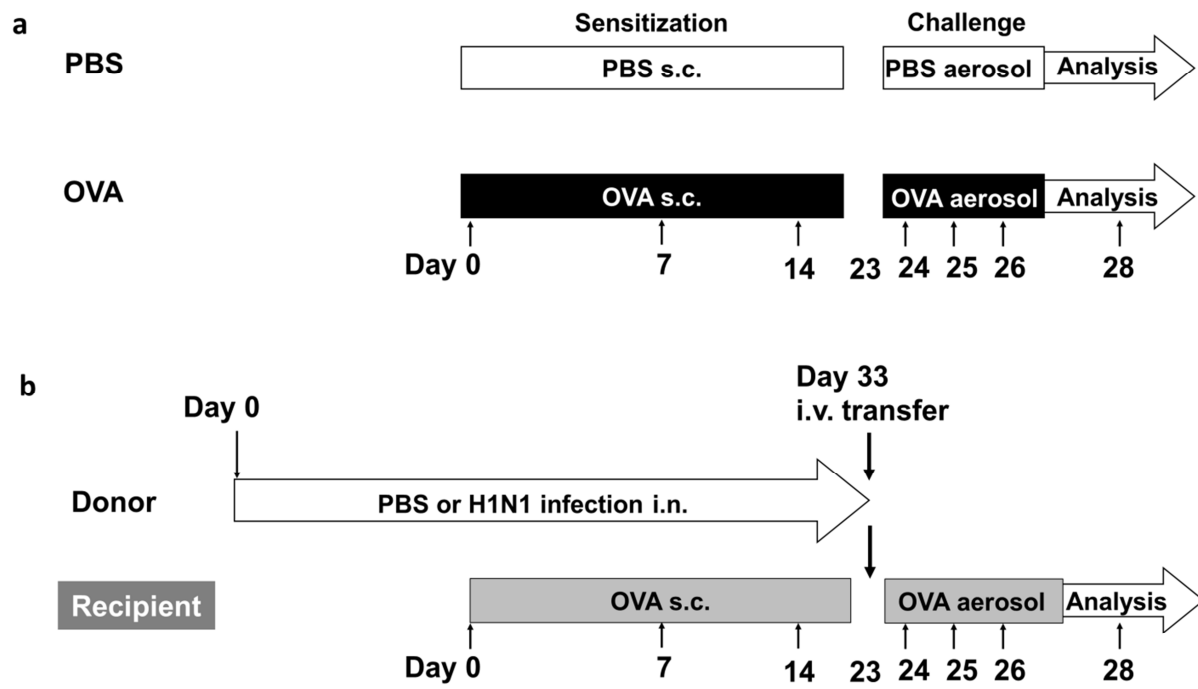


Fig E3.

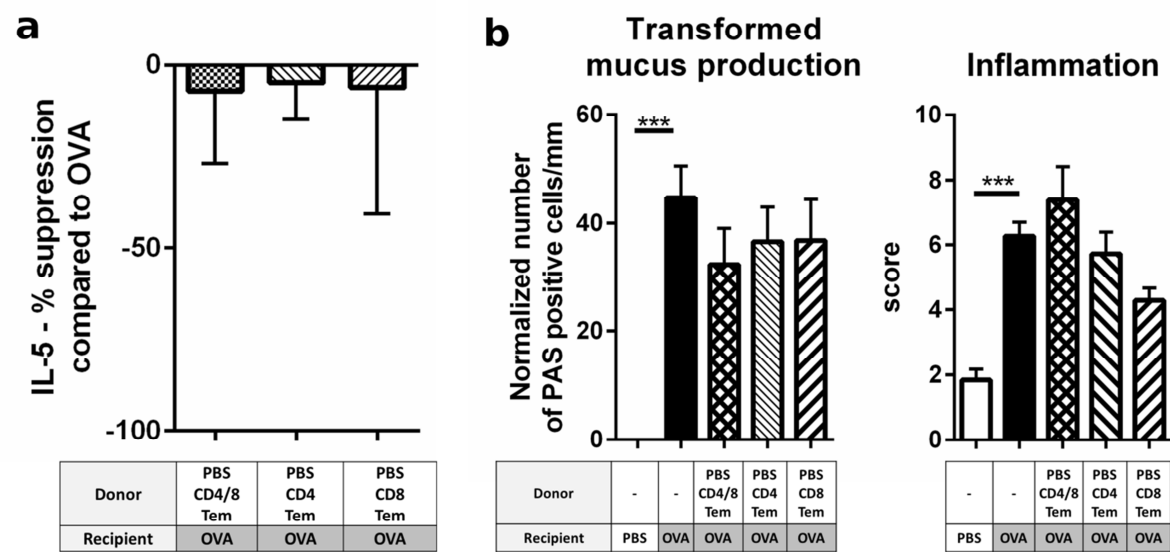


Fig E4.

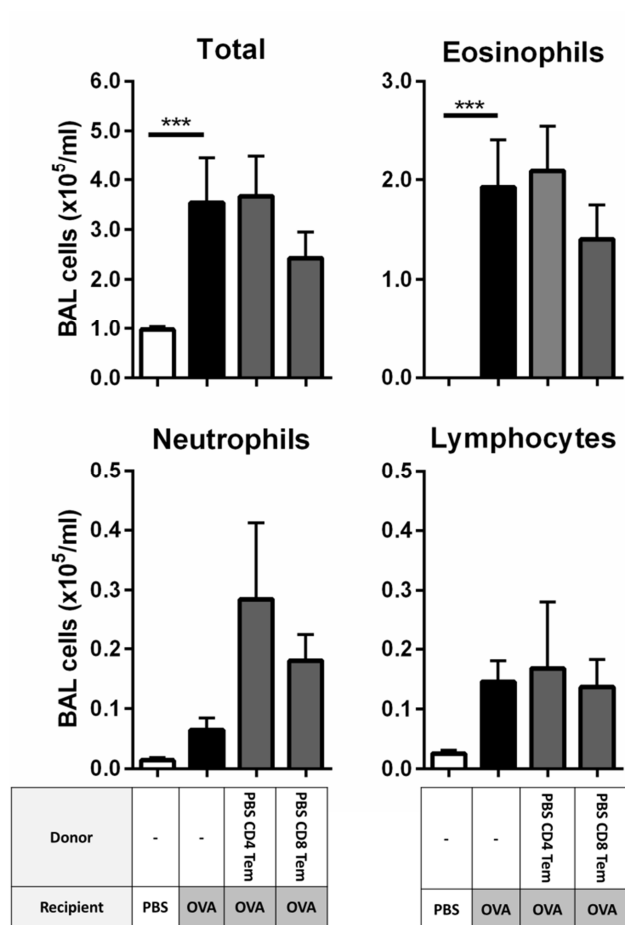


Fig E5.

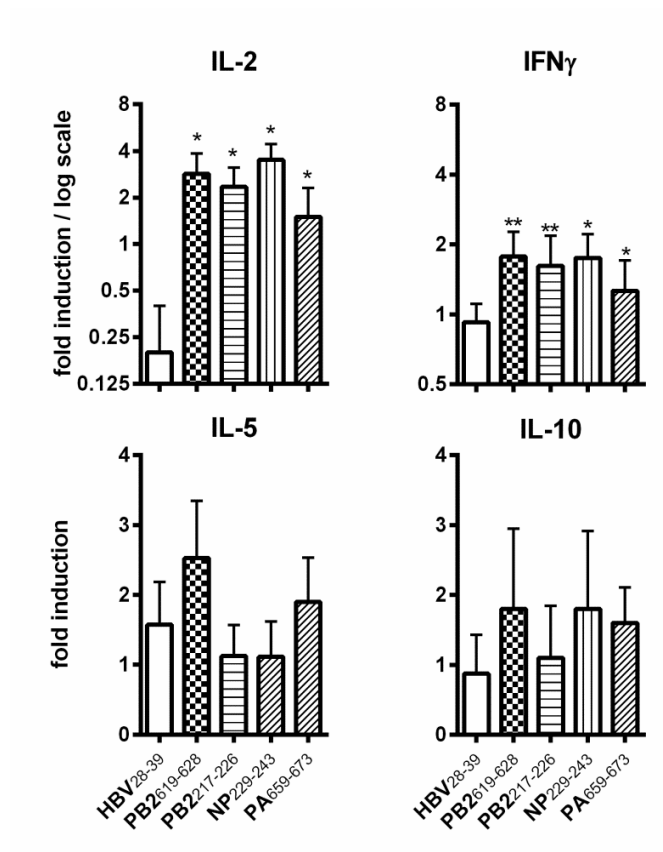


Fig E6.

Table E1.

Peptides (amino acid sequence)										Protein	Amino acid position	e-value						
MHC class I																		
L	P	F	A	A	A	P	P	E	Q	PB2 (H1N1)	619-628	4×10^{-4} (vs OVA ₂₃₃₋₂₄₂)						
L	P	V	A	G	G	T	G	S	V	PB2 (H1N1)	217-226	4×10^{-3} (vs OVA ₂₃₃₋₂₄₂)						
L	P	F	A	S	G	T	M	S	M	OVA	233-242							
MHC class II																		
-	K	F	Q	T	A	A	Q	R	A	M	M	D	Q	V	R	NP (H1N1)	229-243	7×10^{-6} (VS OVA ₁₃₃₋₁₄₇)
I	N	F	Q	T	A	A	D	Q	A	R	-	E	L	I	N	OVA	133-147	
S	A	E	S	R	K	L	L	L	I	V	Q	A	L	R		PA (H1N1)	659-673	2×10^{-3} (VS OVA ₃₁₈₋₃₃₂)
S	A	E	S	L	K	I	S	Q	A	V	H	A	A	H		OVA	318-332	