Supplementary information

Materials and Methods

Immunization protocol

Female C57BL/6 mice (n = 10) were immunized intranasally (i.n.) on day 0, 14, 28 and 62 using different vaccine formulations encompassing 10 µg of ovalbumin (OVA) alone or admixed with 10 µg of either poly[di(sodium carboxylatoethylphenoxy) phosphazene] (PCEP) or poly[di(sodium carboxylatophenoxy) phosphazene] (PCPP), as well as 10 µg of c-di-AMP and 20 µg of IDR in an aqueous solution. As positive control the well-known TLR3 agonist poly I:C (10 µg/dose/animal) was used (Table 1) as it has been shown to promote the induction of effector cytotoxic CD8+ T-cells by facilitating cross-presentation of the antigen which among neutralizing antibodies would be essential in order to efficiently protect against virus infections.[1] Furthermore, Lee et al. demonstrated the protective capacities of poly I:C in mice.[2] While no endotoxins have been detected for c-di-AMP polyphosphazenes were found to have endotoxin levels below 0.034 ng/ml.

Sample collection

After collecting blood samples from mice at specific time points, Sera were gained by incubating blood samples for 1 h at 37°C followed by 30 min incubation at 4°C. Afterwards, samples were centrifuged for 10 min at 3000 × g and sera were stored at −20°C prior to determination of OVA-specific antibodies.

On day 81, mice were sacrificed, broncho-alveolar lavages (BAL), nasal washes and spleens were collected and single cells were obtained by homogenization of the organs mechanically using a sieve, pooled within groups and collectively analyzed for the presence of antigen-specific cells, as described previously.[3]

Detection of antigen-specific antibodies by ELISA

In brief, microtiter plates were coated with 100 µl/well of the antigen (2 µg/ml in 0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4°C. Unspecific binding sites were then blocked incubating the plates with 3% bovine serum albumin (BSA) in PBS for 1 h at 37°C.

Afterwards, plates were washed with 1% BSA/PBS/0.05% Tween 20 and serial 2-fold dilutions of sera in 3% BSA/PBS were added (100 µl/well). After 1 h incubation at 37°C plates were again washed six times with 1% BSA/PBS/0.05% Tween 20 before the secondary antibodies were added: biotinylated goat anti-mouse IgA and IgG, respectively, or for determination of different IgG subclasses, biotinylated goat anti-mouse IgG1 and IgG2c (Sigma, USA), respectively. Subsequently, samples were incubated at 37°C for 1 h. After six washing steps, 100 µl of peroxidase-conjugated streptavidine (BD Pharmingen, USA) was added to each well and the plates were incubated at room temperature (RT) for 1 h. Finally, after another six washes, reactions were developed using ABTS [2, 20-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Endpoint titers were expressed as absolute values of the last dilution that gave an optical density at 405 nm that was 2 times higher than the negative control values after 5 min incubation. To compensate for variations in the efficiency of recovery of secretory antibodies among animals, the results were normalized and expressed as end point titers of antigen-specific IgA per µg of total IgA present in the sample.

Identification of multifunctional T cells and cytokine profiling by FACS staining

Splenocytes ($2x10^7$ cells per well) of immunized mice were incubated (37°C, 5 % CO_2) in 2 ml RPMI containing OVA protein [10µg/ml]. After 20 hours, 5 µg/ml brefeldin A and 6 µg/ml monensin (Sigma-Chemie, Germany) were added and cells were incubated for additional 6 h. Subsequently, cells were stained for dead cells (Fixable Dead Cell Stain, Invitrogen, USA) and surface markers (CD3, CD4 and CD8, BD, USA; CD4, eBioscience, Germany). Then, cells were fixed with 2 % paraformaldehyde, permeabilized for 45-60 min with 0.5% BSA and 0.5% saponin in PBS and stained for intracellular cytokines (IL-2 and IFNγ, BD, USA; TNFα, IL-17, IL-10 and IL-4, eBioscience, Germany). Finally, cells were resuspended in PBS and light emission was measured using BD LSRII. After spectral overlap compensation with the BD FACS Diva Software, the data were analyzed using FlowJo (Tree Star, USA).

Determination of lymphocyte-mediated cytotoxicity *in vivo* (*In vivo* CTL)

Splenocytes [$2x10^7$ cells/ml] of donor mice were incubated for 2 hours at 37°C in the presence of 10 µM OVA peptide (SIINFEKL). Then, cells were stained with 0.5 µM (unpulsed cells) and 5 µM (OVA peptide pulsed) CFSE. After 5 min of incubation at RT staining was blocked by adding FCS. After another 5 min of incubation at RT cells were washed with PBS and adjusted to $1x10^8$ cells/ml. Finally, pulsed and unpulsed cells were mixed in a ratio 1:1 and 200 µl of the cell suspension were injected intravenously into immunized mice (n=3). 48 hours after injection spleen and draining lymph nodes of injected animals were collected and FACS analysis has been performed. The % specific killing was calculated based on the relative ratios of target cells present in immunized mice compared with those in the naïve animals. The ratio between peptide-pulsed to unpulsed target cells in naïve mice is defined as 0% killing. Thus, the following formula was used: % specific killing = (1 – (value peptide-pulsed primed target cells / value unpulsed primed target cells) / (value peptide-pulsed naïve target cells / value unpulsed naïve target cells)) x 100.

Challenge studies

Groups (n=4) of female C57BL/6 (H-2k) were challenged 41 days after the last immunization with a sub-lethal dose ($2x10^3$ FFU) of the influenza strain A/WSN/OVA_I (H1N1). This virus expresses the OVA CD8 epitope OVA257-264 (SIINFEKL) embedded in the neuraminidase protein. In brief, mice were anesthetized by intraperitoneal injection with ketamine/xylazine combination with a dose adjusted to the individual body weight (200 µl/20 g). The virus was administered in a total volume of 20 µl, 10 µl per nostril. Following infection, mice were monitored daily for morbidity during two weeks.

References

1. Garg R, Latimer, L, Simko, E, Gerdts, V, Potter, A and van den Hurk, S. Induction of mucosal immunity and protection by intranasal immunization with a respiratory syncytial virus subunit vaccine formulation. *The Journal of general virology* 2014;95:301-6.
2. Lee S, Stokes, KL, Currier, MG, Sakamoto, K, Lukacs, NW, Celis, E, et al. Vaccine-elicited CD8+ T cells protect against respiratory syncytial virus strain A2-line19F-induced pathogenesis in BALB/c mice. *Journal of virology* 2012;86:13016-24.

3. Ebensen T, Libanova, R, Schulze, K, Yevsa, T, Morr, M and Guzman, CA. Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. *Vaccine* 2011;29:5210-20.