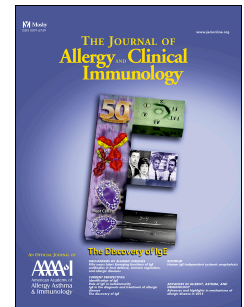


Accepted Manuscript

Rescue of T-cell function during persistent pulmonary adenoviral infection by TLR9 activation

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PII: S0091-6749(17)31282-4

DOI: [10.1016/j.jaci.2017.06.048](https://doi.org/10.1016/j.jaci.2017.06.048)

Reference: YMAI 12963

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 10 January 2017

Revised Date: 6 June 2017

Accepted Date: 29 June 2017

Please cite this article as: Holland T, Wohlleber D, Marx S, Kreutzberg T, Vento-Asturias S, Schmitt-Mbamunyo C, Welz M, Janas M, Komander K, Eickhoff S, Brewitz A, Hasenberg M, Männ L, Gunzer M, Wilhelm C, Kastenmüller W, Knolle P, Abdullah Z, Kurts C, Garbi N, Rescue of T-cell function during persistent pulmonary adenoviral infection by TLR9 activation, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.06.048.

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Rescue of T-cell function during persistent pulmonary adenoviral infection by TLR9 activation

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Funding: Research reported in this publication was supported by DFG Sonderforschungsbereich grant 704 to N.G.. W.K., P.K., Z.A., C.K. and N.G. are members of the DGF ImmunoSensation Cluster of Excellence.

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Capsule summary:

Pulmonary persistent adenoviral infections induce an immunosuppressive environment in the lung that renders CD8 T-cells dysfunctional. Innate cell activation during persistent infection re-invigorates effector T cell function leading to viral clearance and immune homeostasis.

Key words: adenovirus; persistent infection; lung; T cell dysfunction; immunosuppression; immunotherapy; CpG.

To the Editor:

It is being increasingly recognized that the lung harbors persistent subclinical viral infections with unknown effects on immunity. Pulmonary adenoviral infections can become lethal and yet adenoviruses are detected in the lung of otherwise healthy individuals at a relatively high frequency, similar to CMV (1,2), suggesting viral persistence. However, it is currently unknown whether and, if so, how persistent adenoviral infections localized in the lung affect immune responses, because suitable experimental models are lacking. To establish a novel murine model of pulmonary adenoviral persistence, we inoculated intratracheally (i.t.) AdLGO, a recombinant adenovirus coding for luciferase, eGFP and the model antigen ovalbumin, which allows convenient tracking T cell responses.

Inoculation of AdLGO i.t. as described in this article's Online Repository resulted in a dose-dependent infection of the respiratory tract (**Fig 1, A**) that steadily declined in a CD8 T cell-dependent manner until about 16 days post-infection (dpi) (**Fig 1, B**). Beyond that time point, the infectious load after instillation with 5×10^8 infection units (i.u.) remained stable for at least 100 dpi (**Fig 1, C and D**). Most of the persistent infection localized to bronchial and alveolar epithelial cells as demonstrated by confocal microscopy and flow cytometry using a similar adenovirus expressing tdTomato (**Fig 1, E; Fig E1** in this article's Online Repository). Mice displayed normal appearance, behavior and weight (**Fig E2**), indicating a subclinical course of chronic infection.

Although virus-specific CD8 T cells were detected locally at high frequencies (**Fig E3**) and they were clearly protective during the acute phase of AdLGO infection, they failed to fully eradicate infected cells (**Fig 1, B**), suggesting an impairment in their effector function. To test whether a persistent adenoviral infection induced functional

impairment of CD8 T cells, CFSE-labeled AdLGO-specific CD45.1⁺ OT-I CD8 T cells were adoptively transferred into chronically-infected B6 mice that were, in addition, challenged intratracheally with their cognate antigen Ova. Although persistent adenoviral infection did not impair the proliferative response of OT-I CD8 T cells towards the additional Ova challenge, it did inhibit expression of granzyme B (GzmB) (**Fig 1, F**, left *versus* middle panel), a hallmark of CD8 T cell cytotoxic function (3). In addition, OT-I CD8 T cells were also impaired in CD25 expression and their capacity to produce IFN γ , TNF α (Fig E4), indicating a general dysfunction. We next investigated whether the CTL response was impaired also against pulmonary antigens unrelated to the AdLGO infection. For this we challenged AdLGO-infected mice by i.t. instillation of an lymphocytic choriomeningitis virus (LCMV) antigen and then, monitored the immune response of adoptively-transferred P14 CD8 T cells (specific for H2-D^b/LCMV-GP₃₃₋₄₁). Although persistent AdLGO infection did not impact proliferation of P14 cells, it again inhibited GzmB expression (**Fig 1, G**). Collectively, these results indicate that persistent adenoviral infection localized to the lung induces a suppressive environment that impairs acquisition of effector function by pulmonary cytotoxic lymphocytes (CTLs).

Based on these results, we hypothesized that reverting the suppressive inflammatory environment in the lung into a pro-inflammatory setting might promote the accumulation of protective virus-specific effector CTLs. We used CpG-rich immunostimulatory oligodeoxynucleotide (CpG) for this purpose because it was shown to promote protective T cell responses in a different setting of unresolved inflammation elicited by tumor growth (4,5) and, in addition, it is proposed for clinical use as an adjuvant due to its ability to promote protective immune responses (6). CpG i.t.

instillation was able to modulate the pulmonary inflammatory context during chronic AdLGO infection as demonstrated by enhanced expression of type-1 cytokines IFN γ and IL-12 and of other pro-inflammatory cytokines such as TNF α , IL-1 α , IL-6, IL-5, IL-13 and IL-17 in the bronchoalveolar lavage (**Fig E5**). Strikingly, there was a rapid decrease in pulmonary adenoviral load, falling below detection limit within 10 days after onset of CpG immunotherapy (**Fig 2, A**). In contrast, systemic CpG administration did not result in viral clearance (**Fig 2, A**), indicating that modulation of the lung inflammatory context was required for the therapeutic effect.

Depletion of CTLs from chronically-infected mice completely abrogated CpG-induced AdLGO clearance (**Fig 2, B**), demonstrating a crucial role of CTLs in this therapeutic setting. CpG administration i.t. remarkably increased virus-specific CTL numbers in the lung by approx. 60-fold (**Fig 2, C**). This increase in pool size was mediated both by enhanced antigen-specific proliferation in the mediastinal LNs (**Fig 2, D; Fig E6**) and by a reduction in their apoptotic rate in the lung (**Fig 2, E**).

The co-inhibitory receptors Tim-3 and CTL-4 (7) were expressed by virus-specific CTLs during the persistent phase of adenoviral infection independently of CpG application (**Fig E7, A**), indicating that they did not play a dominant role during CpG immunotherapy. Although CpG instillation did result in a partial decrease of PD-1 expression (**Fig E7, B**), our results indicate that this decrease was not sufficient to mediate full viral clearance because blockade of PD-L1 resulted only in a minor, non-statistically significant, decrease in adenoviral signal (**Fig E7, C**).

We next investigated whether local CpG therapy can also improve CTL functionality. Most virus-specific effector CTLs in the lung expressed high levels of GzmB during the AdLGO acute infection (7 dpi) (**Fig 2, F**), consistent with their ability to

eliminate most infected cells (**Fig 1, B**). However, virus-specific CTLs expressed only residual amounts of GzmB during the chronic phase of adenoviral infection (28 dpi) (**Fig 2, F**), consistent with their failure to clear infection. CpG instillation rescued GzmB production, resulting in a large majority of the specific CTLs having regained GzmB expression (**Fig 2, F**). Adenovirus-specific CTLs from CpG-treated mice that had cleared the infection showed even stronger antigen-specific killing than those from acutely-infected mice (**Fig E8**), demonstrating a competent cytotoxic function.

Effector CTL function is characterized by a metabolic bias towards aerobic glycolysis (8). Consistent with a recovered cytotoxic function, CpG application promoted glucose uptake (**Fig 2, G**) and reduced mitochondrial membrane potential in virus-specific CTLs (**Fig 2, H**), suggesting a switch to aerobic glycolysis. Thus, we conclude that CpG instillation had a quantitative and qualitative effect on virus-specific effector CTLs, ultimately allowing to clear persistent adenoviral infection in the lung.

In summary, we present here a novel murine model of subclinical persistent adenoviral infection in the lung that has a profound and detrimental impact on CTL responses. Modulation of the local inflammatory context resulted in recovery of CTL function and elimination of infected cells. Although adenoviral pulmonary infections in patients are common (1,2), the extent of T cell immunosuppression and the responsible mechanisms are not well defined yet due to lack of clinical trials and animal models. The model here presented will be instrumental for delineating the underlying mechanisms and facilitating the development of potential therapeutic strategies for viral clearance from the lung.

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Author contributions: T.H., C.W. and N.G. designed research; T.H., S.M., T.K., S.V.-A., C.S.-M., K.K., and N.G. performed research; D.W., M.W., M.J., S.E., A.B., M.H., L.M., and M.G. contributed new reagents or analytic tools; W.K., P.K., Z.A., and C.K. contributed intellectually; T.H. and N.G. wrote the original draft.

Acknowledgements

We thank Melanie Eichler and the Flow Cytometry Core Facility of the Bonn university hospitals for technical assistance. Funding was obtained from the DFG Sonderforschungsbereich grant 704 to N.G.. W.K., P.K., Z.A., C.K. and N.G. are members of the DGF ImmunoSensation Cluster of Excellence.

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Figure legends

Fig 1. Persistent pulmonary infection in mice with adenovirus results in CD8 T cell functional impairment. **A**, Bioluminescence images of B6-albino mice i.t. infected with the indicated AdLGO doses. **B**, Bioluminescence kinetics in the thorax of AdLGO infected mice. **C**, Bioluminescence images of B6-albino mice i.t. infected with 5×10^8 AdLGO if.u. up to 100 dpi. **D**, Bioluminescence kinetics in the thorax of AdLGO infected mice up to 100 dpi. **E**, Confocal immunofluorescence of lungs infected 28 or 100 days earlier with tdTomato-expressing AdLTO, or AdLacZ as negative control. Infected cells (tdT⁺) are shown in red. Arrowheads, tdT⁺ epithelial cells. Bar = 100 μ m. **F**, Naive or AdLGO chronically-infected mice received CFSE-labeled naive OT-I cells and some mice were in addition challenged with ovalbumin i.t.. Shown are dot plots indicating the percentage of GzmB⁺ within OT-I CD8 T cells in the mediastinal lymph node, and corresponding quantification (right panel), 3 days post transfer. **G**, As in D, except that mice received P14 CD8 T cells and were challenged with LCMVGP₃₃₋₄₁ antigen i.t.. Dot plots show GzmB expression by P14 cells. i.t., intratracheal; if.u., infection units; dpi, days post-infection. AdLGO, adenovirus encoding for luciferase, eGFP and ovalbumin; AdLTO, adenovirus encoding for luciferase, td-Tomato (tdT) and ovalbumin; GzmB, granzyme B. Shown is a representative of 3 independent experiments (n=4 mice per group). *, p<0.05, ANOVA (F) or Student's *t*-test (G).

Fig 2. Local CpG instillation results in functional recovery of virus-specific CD8 T cells and clearance of persistent adenoviral infection. **A**, Bioluminescence signal in the thorax of chronic AdLGO mice undergoing PBS or CpG treatment (arrows). Mice had been infected with 5×10^8 if.u. on day 0. **B**, As in (A), but some mice received CD8 T cell-depleting antibody (arrowheads). **C**, Percentage (dot plots) and number (right panel) of

1 virus-specific CD8 T cells in the lung of mice treated or not with CpG i.t. as in (A). Dex,
2 K^b/SIINFEKL dextramer. **D**, Proliferation of virus-specific CD8 T cells in the mediastinal
3 lymph node from AdLGO-infected mice and treated as in (A). **E-H**, Apoptotic rate (E),
4 granzyme B expression (F), glucose uptake (G), and mitochondrial membrane potential
5 (H) of pulmonary AdLGO-specific CD8 T cells after CpG or PBS i.t. treatment as in (A).
6 Shown is a representative of at least 3 independent experiments (n=4-5 mice per
7 group). *, p<0.05; **, p<0.01; ***, p<0.001, Student's *t*-test (A, against CpG i.v.; B,
8 against a□□□-CD8α; C; D; E; G; H) or ANOVA (F).

Figure 1 2-column Holland *et al*

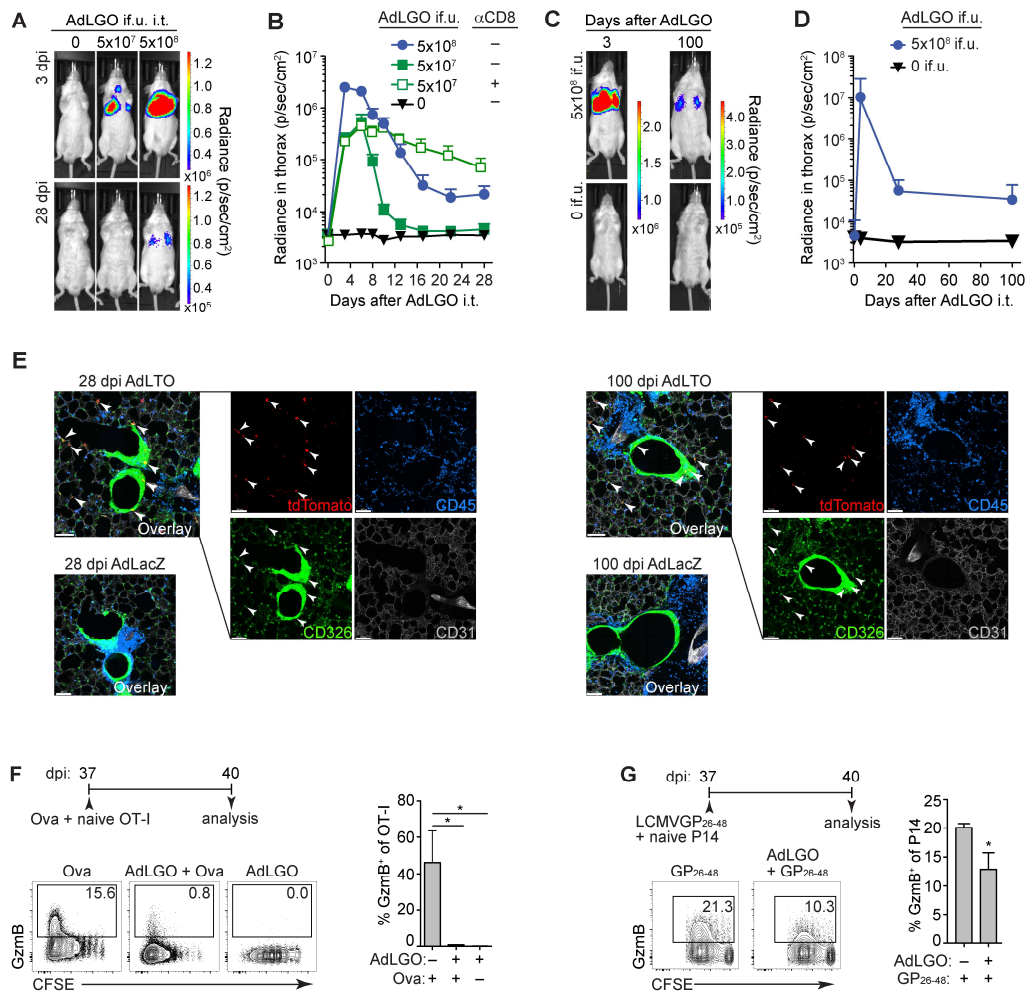
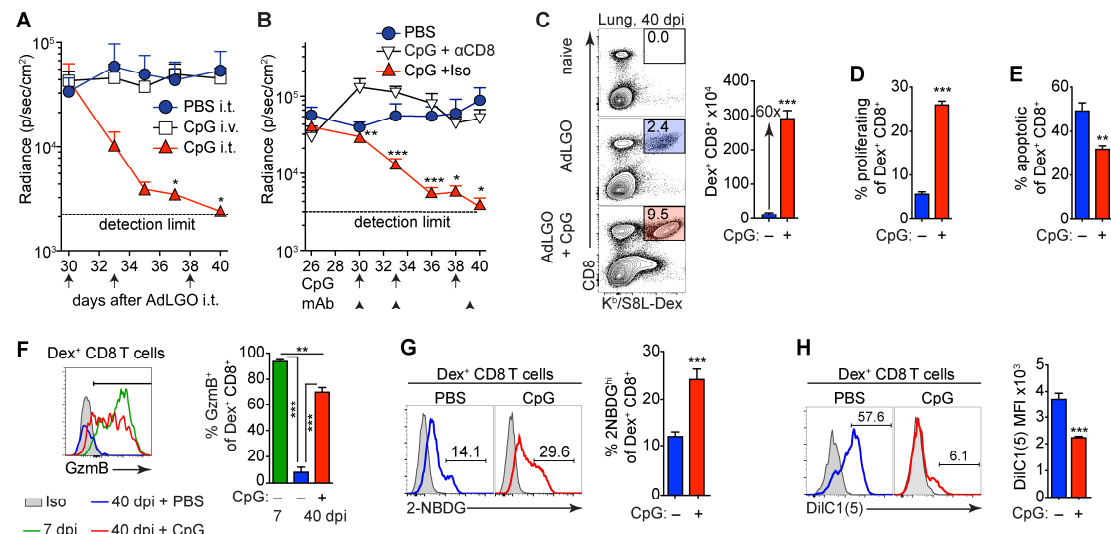


Figure 2 2-column Holland *et al*



METHODS

Mice and adenoviral infection

All mice were on a C57BL/6 background. C57BL/6J (B6), B6-albino (B6(Cg)-*Tyr*^{c-2J}/J) (1), CD45.1 OT-I (2) and P14 (3) mice were bred and maintained under SPF conditions at the animal facility of the University of Bonn Hospitals. OT-I mice harbor CD8 T cells carrying a transgenic TCR that recognizes the ovalbumin-derived epitope SIINFEKL in the context of H-2K^b. P14 mice harbor CD8 T cells carrying a transgenic TCR that recognizes the LCMV glycoprotein-derived epitope KAVYNFATC in the context of H-2D^b. Experiments were performed with sex- and age-matched mice following the institutional and governmental guidelines of animal welfare.

Recombinant adenoviruses

Recombinant adenoviral vectors deleted of the early-transcribed regions E1 and E3 were as follows: AdLGO (expressing click-beetle luciferase CBG99, eGFP and ovalbumin fragment containing the CD4 T and CD8 T cell immunodominant epitopes under the CMV promoter) (4), AdLTO (eGFP from AdLGO replaced by tdTomato) or AdlacZ (expressing *lacZ* β -galactosidase under the CMV promoter). The indicated cDNAs were separated by P2A linker sites from the Porcine Teschovirus 1 followed by bGH poly(A) signal. Recombinant adenoviruses were generated using the Gateway® technology from ThermoFisher. Expression cassettes were cloned into Gateway® pENTR™11 Dual Selection Vector (ThermoFisher Scientific, Germany) and recombined into pAD/PL-DEST™ Gateway® Vector (ThermoFisher Scientific, Germany) *in vitro* via the LR Clonase® Enzyme Mix (ThermoFisher Scientific,

Germany). The resulting adenoviral DNA was propagated on human embryonic kidney (HEK 293) cells and purified according to standard protocols based on cesium chloride density-gradient centrifugation as described (5).

Adenoviral infection and intratracheal instillation

For i.t. administration of adenoviruses (5×10^8 i.f.u. unless stated otherwise), unmethylated CpG-containing oligodeoxynucleotides #1668 (5 μ g; TIB MOLBIOL), ovalbumin (100 μ g, Sigma-Aldrich), extended LCMV GP₂₆₋₄₈ peptide containing the minimal GP₃₃₋₄₁ epitope LIVITGIKAVYNFATCGIFAFTL (25 μ g, Xaia Custom Peptides) or PBS, mice were anesthetized with 3% isoflurane/O₂ (v/v) and intubated with a blunted indwelling 22G x 1" cannula (BBraun) with the help of a light-assisted rodent-laryngoscope (Penn-Century). Subsequently, 50 μ l solution was inoculated and mice were actively ventilated for further 60 seconds with a MiniVent mechanical ventilator (Harvard Apparatus) using 250 μ l tidal volume and 250 strokes/minute.

Bioluminescence imaging

Measurement of AdLGO bioluminescence was performed with an IVIS Lumina system (Caliper Life Science) as previously described (6) by injecting 4.5 mg D-Luciferin i.p.. Mice were kept at 37°C for 5 min and then bioluminescence was measured for 120 sec at 37°C. In some experiments, following *in vivo* bioluminescence imaging organs were dissected out, incubated in 4.5 mg/ml D-Luciferin at 37°C for 5 minutes and imaged *ex vivo* at 37°C. Image analysis was performed with Living Image version 4.3.1. (Caliper Life Science) by defining a region of interest in the thorax region equal for all mice.

Cell isolation and adoptive CD8 T cell transfer

Organs were dissected and disrupted in DPBS containing 3% FCS, 1 mg/ml collagenase IV (Sigma-Aldrich) and 50U/ml DNase I (Roche) for 30 min at 37°C. Cell suspensions were then filtered and washed twice in PBS at 4°C. BALF was obtained by three consecutive airway washes with 1 ml PBS containing 0.1 mM EDTA. For adoptive transfer of naive CFSE-labeled OT-I and P14 cells, splenic CD8 T cells were positively selected with CD8 microbeads (Miltenyi Biotec), labeled with 1 μ M CFSE and transferred i.v. (7). 1×10^6 CD8 T cells were transferred i.v. into mice that had been infected with 5×10^8 i.f.u. AdLGO 37 days earlier. Analysis was performed in mediastinal lymph nodes 3 days after transfer.

In vitro restimulation of CD8 T cells

$10^5 - 10^6$ cells per well were restimulated *in vitro* in 96-U wells in 200 μ l RPMI containing 50 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin, GolgiPlug/GolgiStop (BD Biosciences) for 5 h at 37°C and 5 % CO₂. For degranulation analysis, BV421-labeled mAb 1D4B directed against CD107a (Biolegend) was added at a 1/100 dilution during the 5h restimulation *in vitro*. Cells were then washed twice and immediately processed for flow cytometric analysis.

Flow cytometry and cell sorting

Flow cytometry of viable cells was performed using standard techniques. Antibodies directed against the following antigens were used: CD8 α (53-6.7), CD8 β (YTS156.7.7), CD45.2 (104), CD45.1 (A20), CD4 (GK1.5), CD11b (M1/70), CD11c (N418), CD19 (6D5), CD44 (IM7), CD326 (G8.8), CD31 (MEC13.3), F4/80 (BM8), FoxP3 (FJK-16s), granzyme B (GB11), I-A/I-E (M5/114.15.2), CD64 (X54-5/7.1),

KLRG1 (2F1/KLRG1), F4/80 (BM8), Ly6-C (HK1.4), Ly6-G (RB6-8C5), NK1.1 (PK136), CD3 (145-2c11), CD115 (AFS98), PD-1 (RMP1-30), Tim3 (B8.2C12), CTLA-4 (UC10-4B9), CD160 (7HI), KLH (RTK2071, RTK4530) (all from BioLegend), and Siglec-F (E50-2440) (eBioscience).

For Dextramer staining, cells were stained with K^b/SIINFEKL-Dextramer (Immudex) prior to antibody staining in PBS containing 5% FCS for 20 min at 4°C.

For intracellular staining, the Foxp3/Transcription factor buffer set (eBioscience) was used according to the manufacturer's guidelines.

Annexin V staining was done with the Annexin V apoptosis detection kit (BioLegend) according to the manufacturer's guidelines. Following Annexin V staining, cells were stained with K^b/SIINFEKL-Dextramer in Annexin V-binding buffer containing 5% FCS for 20 min at 4°C.

For glucose uptake assay, freshly isolated cells were cultured with 10 µg/ml 2-NBDG (Cayman Chemicals) in glucose free RPMI for 15 min at 37°C, stained for surface markers and analysed for 2-NBDG uptake by flow cytometry.

The mitochondrial membrane potential was determined by using the MitoProbe DiIC1(5) Assay Kit (ThermoFisher Scientific) and the total mitochondrial mass was evaluated with the MitoTracker Deep Red Kit (ThermoFischer Scientific) according to the manufacturer's guidelines.

For staining of DNA-content, after fixation in 4 % methanol-free paraformaldehyde for 20 min at 4°C, cells were resuspended in FoxP3/Transcription factor permeabilization buffer (eBioscience) containing 3 µM DAPI (BioLegend), incubated for 15 min at RT, and immediately analyzed by flow cytometry.

Dead cells were excluded by fixable viability dye eFluor 780 (eBioscience), Hoechst 33342 (Sigma-Aldrich) or propidium iodide (Sigma-Aldrich).

Samples were acquired on an LSR Fortessa (BD Biosciences) or FACSCanto II (BD Biosciences) and analyzed with FlowJo version 10.1 (TreeStar). Cell sorting was performed on a FACS Aria with cooling at 4°C.

Confocal microscopy

Vibratome sections were used to visualize AdLTO-infected cells. Briefly, lungs were kept in the inspiration phase by inflating them with 1 ml 2% low-melting agarose solution (Promega) at 37°C. Lungs were dissected out, placed at 4°C for 30 min and subsequently embedded in 4% low-melting agarose solution. Embedded lungs were cut with a Leica VT1000S vibrating-blade microtome at 100 µm thickness. Sections were kept on ice and immediately processed for staining without fixation to preserve tdTomato fluorescence by blocking in PBS containing 3% FCS and 15 mg/ml human poly-immunoglobulin (Privigen) for 30 min at 4°C. Sections were then stained with antibodies in PBS containing 3% FCS for 16 h at 4°C and washed 4 times in PBS. The following antibodies were used: anti-CD45-brilliant violet 421 (30-F11), anti-CD326-Allophycocyanin (APC) (G8.8), and anti-CD31-Fluorescein isothiocyanate (MEC13.3) (BioLegend). Sections were mounted in PBS and immediately analyzed on a 710 confocal microscope (Carl Zeiss Microimaging). Data were analyzed with Imaris version 7.6.3 (Bitplane).

Cytokine profiling

Cytokines in BALF were quantified using the Mouse Th1/Th2 10-plex flow cytometric bead assay (eBioscience) or a custom-made mouse 7-plex bead assay (BioLegend), according to the manufacturer's guidelines. For this, mice were infected with 5×10^8

AdLGO if.u. i.t. and treated or not with 5µg CpG at the indicated times. The BAS was lavaged with 1 ml PBS and the BALF frozen at -70°C until analysis.

***Ex vivo* cytotoxicity assay**

For antigen-specific kill activity of CTLs, total leukocytes were isolated by lavaging the BAS of mice infected with 5×10^8 AdLGO if.u. i.t. either 7 days earlier (acute infection) or 40 days earlier plus 5µg CpG i.t. 30, 33, and 38 dpi. Effector K^b/S8L-specific CTLs were adjusted to equal numbers in the different effector samples by staining an aliquot with the respective dextramer. Specific target cells were generated by pulsing B6 splenocytes with 10 µM SIINFEKL (Xaia Custom Peptides) peptide for 30 min and further labeling with 3 µM cell proliferation dye eFluor 670 (eBioscience) for 15 min (eF^{hi}) at room temperature. Non-specific target cells were unpulsed B6 splenocytes and labeled with 0.3 µM of cell proliferation dye eFluor 670 (eF^{lo}). Specific and non-specific target cells were mixed at a 1:1 ratio. BALF CTLs were then mixed with 2×10^4 total target cells in a V-bottom 96-well plate (CELLSTAR) at different effector:specific target ratios (4:1, 1:1, 0.2:1; maximum number of K^b/S8L-specific CTLs was 4×10^4). As a negative control, target cells were incubated without CTLs. Specific cytotoxicity was calculated 5 h later by flow cytometry using the following equation: $100 - [100 \times (eF^{hi}/eF^{lo})_{+CTLs} / (eF^{hi}/eF^{lo})_{-CTLs}]$.

Antibody treatment in vivo

In vivo depletion of CD8 T cells was performed by i.p. administration of 300 µg YTS169.4 (8) antibody (BioXCell), respectively, every 3-6 days. A respective concentration of anti-KLH mAb (LTF-2, BioXCell) was used as isotype control.

For blockade of PD-1–PD-L1 axis, 200 µg anti-PD-L1 mAb (10F.9G2, BioXCell) (9) was administered i.p. every 2-3 days. A respective concentration of anti-KLH mAb (LTF-2, BioXCell) was used as isotype control.

Statistical analysis

Statistical significance was set at $P < 0.05$ and determined by a two-tailed unpaired Student's *t*-test or a one-way ANOVA test as appropriate using GraphPad Prism 5.0a software (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data are expressed as mean $\pm$ SEM. Data shown are representatives of at least three experiments with three to six mice per group.

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Supplemental Figure Legends

Fig E1. Pulmonary epithelial cells constitute the main cellular compartment infected with adenovirus during the chronic phase.

Representative contour plots showing the percentage of infected cells (tdTomato⁺-tdT⁺) in lungs of mice instilled 28 days earlier with 5×10^8 i.f.u. i.t. tdTomato (tdT)-expressing AdLTO, or AdlacZ as negative control. Gating was done as following: EpC, epithelial cells (CD326⁺CD31⁻CD45.2⁻Ter119⁻); EnC, endothelial cells (CD326⁻CD31⁺CD45.2⁻Ter119⁻); O.S., other stromal cells (CD326⁻CD31⁻CD45.2⁻Ter119⁻); AM, alveolar macrophages (Siglec-F⁺CD11c⁺CD45.2⁺); non-AM leukocytes (Siglec-F⁻CD45.2⁺), iM, interstitial macrophages (Siglec-F⁻CD45.2⁺CD11c⁻CD11b⁺F4/80⁺) and DC, dendritic cells (Siglec-F⁻CD45.2⁺CD11c⁺I-A/I-E⁺). All events were pre-gated on live cells. Bar graphs show quantification of data in the contour. MFI, mean fluorescence intensity. Shown is a representative of three independent experiments. ***, $p > 0.001$, ANOVA.

Fig E2. Body weight progression in mice undergoing pulmonary adenoviral infection.

Fig E3. Adenovirus-specific CD8 T cells are generated during the acute phase of infection.

A, B6 mice were infected i.t. with 5×10^8 AdLGO i.f.u. i.t. and the frequency and number of K^b/SIINFEKL-specific CD8 T cells identified by dextramer staining in the indicated samples. Flow cytometry density contours were pre-gated on live CD8 T cells. N.D., not detected (CD8 T cells are not present in the bronchoalveolar space of naive mice); mLN, lung-

draining mediastinal lymph nodes. Dex, dextramer; $K^b/S8L^+$, $K^b/SIINFEKL$ -Dex⁺. **B**, Frequency of total CD8 T cells in the indicated samples of naive and AdLGO infected mice as in (A). Shown is a representative of at least three independent experiments.

Fig E4. Adenovirus-specific CD8 T cells fail to upregulate effector molecules and mechanisms during adenoviral persistence. AdLGO infection, ovalbumin (Ova) challenge i.t. and adoptive transfer of CD45.1+ CFSE-labelled AdLGO-specific naive OT-I T cells were performed as in Fig. 1, F. Analysis of CD25 (**A**), IFN γ (**B**) and TNF α (**C**) expression as well as degranulation activity (**D**) by OT-I CD8 T cells in the mediastinal LN was performed 3 days after transfer. Shown is a representative of 3 independent experiments (n=4 mice per group). *, p<0.05; **, p<0.01; ***, p<0.001; ANOVA (n=4 mice per group).

Fig E5. CpG instillation i.t. promotes expression of pro-inflammatory cytokines in the lungs of mice with AdLGO persistent infection. Quantification of cytokine content in the BALF of mice infected or not with 5×10^8 AdLGO i.f.u. i.t. (**A**) Mice were treated on 38 dpi with PBS or 5 μ g CpG i.t. and the BAL was collected 48h later. (**B**) naive mice, mice infected with 5×10^8 AdLGO i.f.u. 7 days earlier, or 40 days earlier and treated with PBS or 5 μ g CpG i.t. on 30, 33 and 38 dpi. BAL was collected 48h later. Shown is a representative of 2 independent experiments (n=4 mice per group). *, p<0.05; **, p<0.01; ***, p<0.001; ANOVA.

Fig E6. Enhanced virus-specific CTL proliferation following local CpG instillation in AdLGO persistently infected mice. Histograms (left panels) showing % of proliferating CD44^{hi}K^b/S8L-Dex⁺CTLs in chronically infected mice recovered from lung or mediastinal LN (mLN). AdLGO, infected with 5×10^8 if.u. AdLGO 40 days earlier and treated with PBS i.t. on 30, 33 and 38 dpi; AdLGO + CpG, similarly infected and treated with 5 μ g CpG i.t. Right panel, respective cumulative analysis for Dex⁺ and Dex⁻ CD44^{hi}CD8 T cells as indicated. Dex, K^b/SIINFEKL dextramer. Shown is a representative of 3 independent experiments (n=4 mice per group). **, p<0.01; ***, p<0.001; Student's *t*-test.

Fig E7. Expression of inhibitory receptors on virus-specific CD8 T cells during adenoviral persistence. (A, B) Expression of Tim3, CTLA4 and CD160 (A) and of PD-1 (B) on K^b/SIINFEKL dextramer⁺ (Dex⁺) CD8 T cells in the lung of infected mice having received or not 5 μ g CpG i.t. as in Fig 2 A. (C) Mice were infected with 5×10^8 AdLGO if.u. i.t. and treated with anti-PD-L1 antibodies intraperitoneally (i.p.) when indicated (arrows). Iso, isotype control. Shown is a representative of two experiments using 5 mice per group. ***, p<0.01 (Student's *t*-test).

Fig E8. CpG treatment leads to potent cytolytic activity of virus-specific CTLs in airways. *Ex vivo* K^b/SIINFEKL-specific cytotoxic activity against splenocyte target cells by CTLs isolated from the BALF of either mice infected with 5×10^8 if.u. AdLGO 40 days earlier and treated with CpG i.t. on 30, 33 and

38 dpi, or mice infected with 5×10^8 i.f.u. AdLGO 7 days earlier. E, K^b /SIINFEKL-specific CTLs; T, K^b /SIINFEKL-loaded splenocyte targets.

ACCEPTED MANUSCRIPT

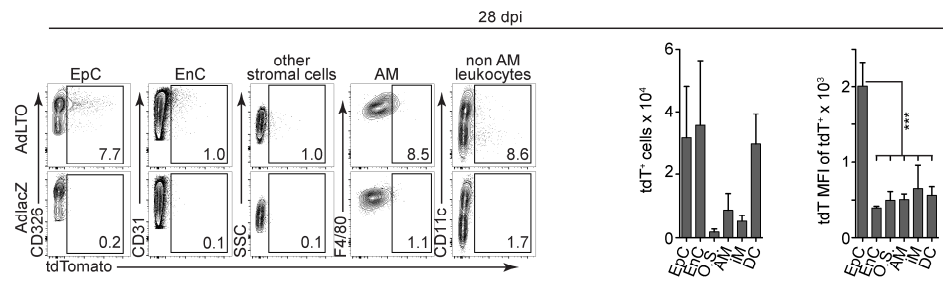
Fig E1 Holland *et al*

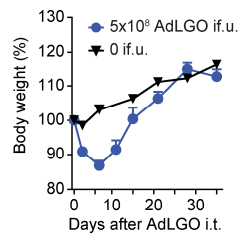
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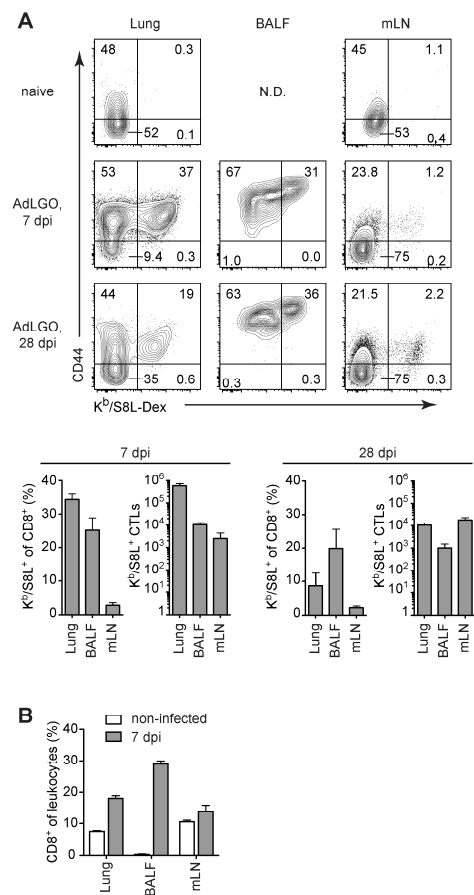
Fig E3 Holland *et al*

Fig E4 Holland *et al*

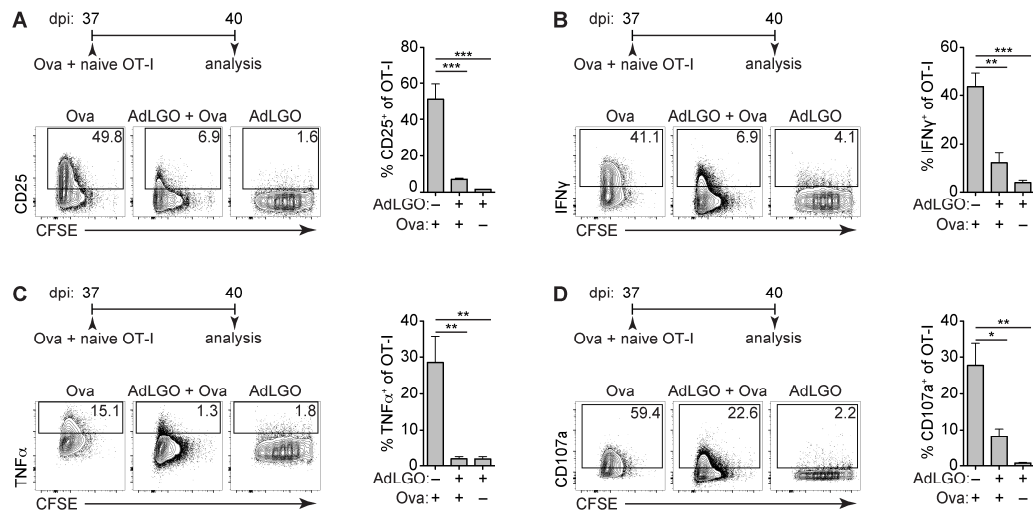


Fig E5

Holland *et al*

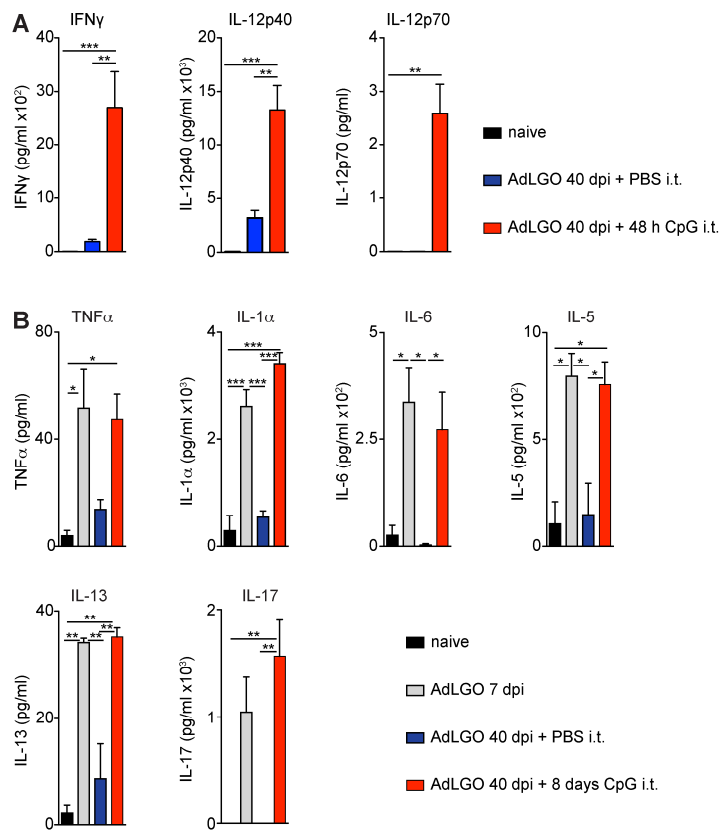


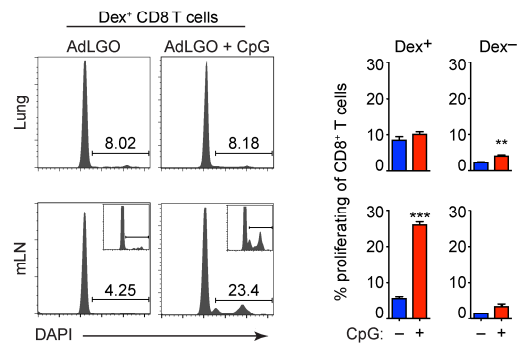
Fig E6 Holland *et al*

Fig E7 Holland *et al*

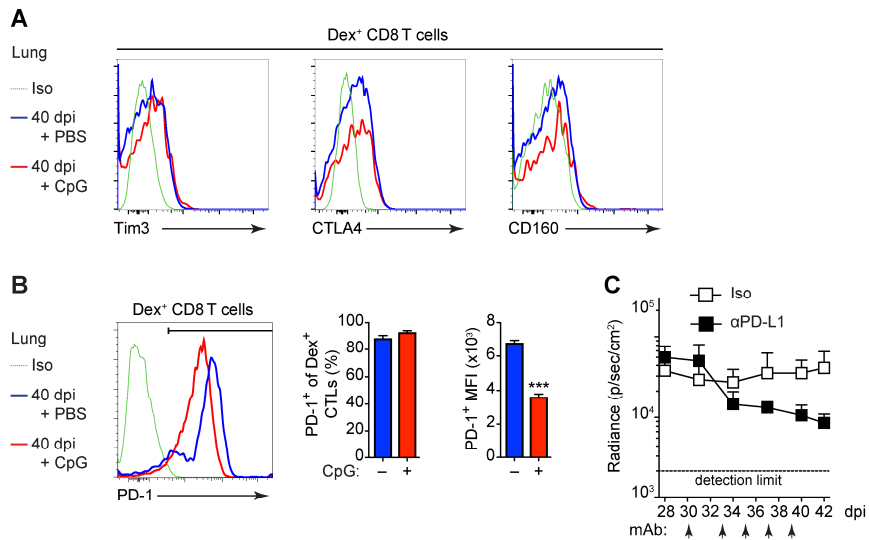


Fig E8 Holland *et al*