Highly Pathogenic Influenza Virus Infection of the Thymus Interferes with T Lymphocyte Development

Annette B. Vogel, Emanuel Haasbach, Sarah J. Reiling, Karoline Droebner, Karin Klingel and Oliver Planz

*J Immunol* 2010; 185:4824-4834; Prepublished online 22 September 2010;
doi: 10.4049/jimmunol.0903631
http://www.jimmunol.org/content/185/8/4824

Supplementary Material  
http://www.jimmunol.org/content/suppl/2010/09/20/jimmunol.0903631.DC1

References  
This article cites 48 articles, 22 of which you can access for free at:  
http://www.jimmunol.org/content/185/8/4824.full#ref-list-1

Subscription  
Information about subscribing to The Journal of Immunology is online at:  
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Highly Pathogenic Influenza Virus Infection of the Thymus Interferes with T Lymphocyte Development

Annette B. Vogel,* Emanuel Haasbach,* Sarah J. Reiling,* Karoline Droebner,* Karin Klingel,† and Oliver Planz*

Highly pathogenic avian influenza viruses (HPAIVs) cause severe disease in humans. Still, the basis for their increased pathogenesis remains unclear. Additionally, the high morbidity in the younger population stays inexplicable, and the recent pandemic H1N1v outbreak in 2009 demonstrated the urgent need for a better understanding about influenza virus infection. In the present study, we demonstrated that HPAIV infection of mice not only led to lung destruction but also to functional damage of the thymus. Moreover, respiratory dendritic cells in the lung functioned as targets for HPAIV infection being able to transport infectious virus from the lung into the thymus. The pandemic H1N1 influenza virus was able to infect respiratory dendritic cells without a proper transport to the thymus. The strong interference of HPAIV with the immune system is especially devastating for the host and can lead to lymphopenia. In summary, from our data, we conclude that highly pathogenic influenza viruses are able to reach the thymus via dendritic cells and to interfere with T lymphocyte development. Moreover, this exceptional mechanism might not only be found in influenza virus infection, but also might be the reason for the increased immune evasion of some new emerging pathogens. The Journal of Immunology, 2010, 185: 4824–4834.

Influenza A virus infection causes severe disease in humans and is a major topic in clinical health. Although epidemic outbreaks of human influenza viruses cause annually over <300,000 deaths worldwide (1), the recent swine origin influenza virus (SOIV) H1N1v pandemic outbreak demonstrates the full threat of influenza virus infection. So far, SOIV infection in humans worldwide causes rather mild clinical symptoms, though >18,000 deaths in humans have been reported (World Health Organization). In contrast, infection with highly pathogenic avian influenza virus (HPAIV) is fatal to humans. Until today, 499 confirmed cases have been reported mainly in southeast Asia, and 295 of them were lethal (World Health Organization). This fatality rate of ~59% demonstrates the urgent need to get a closer insight into the pathogenesis of HPAIV infections in humans, because H5N1 influenza virus is still considered as a potential pandemic strain. Moreover, a reassortant between H5N1 and the 2009 pandemic H1N1v influenza virus might be a candidate for a serious pandemic influenza virus strain with high morbidity and more devastating high fatality rate.

Although annual epidemic outbreaks typically affect people with an impaired immune system (e.g., elderly, infants, and immunosuppressed people), severe influenza after HPAIV infection is mostly found in young and middle-aged people (2, 3). Human infection cluster of H5N1 outbreaks with an average age of 13.7 and 15.4 y have been reported (4, 5). The same phenomenon was found for the Spanish Flu pandemic (6) and the 2009 pandemic H1N1 strain (7). The reason for this altered host specificity remains unclear. One hallmark of H5N1 infection in humans is a strong reduction of lymphocytes, also known as lymphopenia. Lymphopenia is a common feature of some infectious diseases, particularly with new emerging pathogens (8, 9). The mechanism of lymphopenia induced after H5N1 influenza virus infection is largely unknown. Remarkably, H5N1 influenza virus seems to reduce the effector function of CD8+ T cells by an insufficient perforin expression (10). Moreover, it was reported that H5N1 viruses are able to infect cells of the immune system, leading to an impaired adaptive immune response (11). As a consequence, the unbalance between innate and adaptive immune response might lead to a second hallmark of H5N1 infection, namely hypercytokinemia, which is an increase of proinflammatory cytokines and chemokines in the lung (1).

The thymus, where T cell development takes place, presents an organ that is highly active during childhood, but remains functional in young adults. The human thymus has its highest activity phase in an age group from 0–10 y and shows a major activity drop in the population older than 39 y. Nevertheless, the thymus remains still functional in young adults. The human thymus has its highest activity phase in an age group from 0–10 y and shows a major activity drop in the population older than 39 y. Nevertheless, the thymus remains still active in the elderly population (12, 13).

Bone marrow–originated common lymphoid progenitor cells reach the cortical area of the thymus and become TCR+CD4+CD8+ thymocytes. At this stage, the positive selection takes place. During this process, thymocytes with a TCR that does not recognize self-MHC get lost. Surviving thymocytes migrate into the medulla of the thymus where self-peptides are presented. Thymocytes that recognize Ags undergo apoptotic processes and get eliminated.

*Friedrich-Loeffler-Institute, Institute of Immunology and †Department of Molecular Pathology, University Hospital, Tuebingen, Germany

Received for publication November 9, 2009. Accepted for publication August 18, 2010.

This work is part of the EUROFLU consortium activities and the VIRGIL European Network of Excellence on Antiviral Drug Resistance supported by a grant (LSHMCT-2004-503359) from the Priority 1 Life Sciences, Genomics and Biotechnology for Health program in the 6th Framework Program of the European Union. This work was partially supported by the Federal Government of Germany under the Forschung Zoonose program “FluResearchNet”.

Address correspondence and reprint requests to Dr. Oliver Planz, Friedrich-Loeffler-Institut, Institute of Immunology, Federal Research Institute for Animals Health, Paul-Ehrlich Straße 28, D-72076 Tuebingen, Germany. E-mail address: oliver.planz@ flt.bund.de

The online version of this article contains supplemental material.

Abbreviations used in this paper: C, cortex; DC, dendritic cell; ffu, focus forming unit; HPAIV, highly pathogenic avian influenza virus; J, corticomedullary junction; LN, lymph node; M, medulla; MDCK, Madin-Darby canine kidney; MLD50, 50% mouse lethal dose; NP, nucleoprotein; qRT-PCR, quantitative real-time RT-PCR; RDC, respiratory dendritic cell; SOIV, swine origin influenza virus; Treg, regulatory T cell.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00
Only thymocytes that do not recognize MHC class I or II-presented self-peptide become naive T lymphocytes and migrate into periphery. During the highly active life phase, the T lymphocyte production rate of the thymus ranges from $2 \times 10^6$ to $1.8 \times 10^{10}$ cells/d in humans (14). Macrophages and dendritic cells (DCs) in the thymus are also involved in the process of negative selection. It used to be a given fact that both cell types have their origin in the bone marrow, and migration out of the thymus is a one-way event. However, recent reports demonstrated that DCs with extrathymic origins also migrate from the periphery into the thymus (15). The uptake of foreign Ags by DCs and the transport of these Ags into the thymus result in a development of a T cell tolerance against foreign Ags (16).

Fatal H5N1 influenza virus infection in mice results in massive lung damage originated by infiltrating immune cells, namely macrophages and neutrophiles (17). T lymphocytes only play a secondary role in this damage. Moreover, CD8+ T lymphocytes have a poor cytotoxic profile after influenza virus infection (18), and lymphopenia can be observed (1). We hypothesize that the influenza virus itself plays an active role in these findings. A conceivable mechanism could be the direct interference with the thymus as the primary lymphoid organ for T lymphocyte development.

In the present study, we were able to demonstrate that highly pathogenic influenza viruses abuse the homing process of DCs into the thymus after infecting respiratory DCs (RDCs) in the lung. RDCs migrated into the thymus postactivation by the infection with HPAIV, and the viral spread into the thymus interferes with the T lymphocyte selection processes. That way, a reduced cellular immune response against the invading pathogen and the predominantly increased disease symptoms in younger people might be explained. Moreover, the strong atrophic process of the thymus observed during HPAIV infection is a common feature of severe diseases like AIDS, rabies, and Chagas disease (19). Our results give rise to the assumption that this mechanism of increased pathogenesis is not only due to influenza viruses with pandemic potential, but also to infections with other new or re-emerging pathogens like foot-and-mouth disease virus, Chikungunya, severe acute respiratory syndrome, and West Nile Virus (20–24).

Materials and Methods

Mice

Inbred C57BL/6 and C57BL/6-Tg (ACTB-EGFP)1Osb/J (C57BL/6-GFP+) mice at the age of 8–10 wk were obtained from the animal breeding facility at the Institute of Virology, Justus-Liebig University, Giessen, Germany, and were used throughout all the experiments. The C57BL/6-Tg (ACTB-EGFP)1Osb/J mouse strain was originally obtained from Charles River Laboratories (Sulzfeld, Germany).

Virus and infection

Two different highly pathogenic avian influenza virus strains were used throughout this study. The mouse-adapted avian influenza A/FPV/Bratislava/79 (H7N7) virus was grown on Madin-Darby canine kidney (MDCK) cells. The Bratislava strain of the H7N7 avian influenza A virus was originally obtained from the Institute of Virology, Justus-Liebig University, Giessen, Germany. The avian influenza A H5N1 virus A/Mallard/Bavaria/1/2006 grown in embryonated chicken eggs was originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany. The H5N1 strain, the low pathogenic avian influenza virus strain A/Mallard/Bavaria/1/2005 (H5N2) was also obtained from the Bavarian Health and Food Safety Authority and propagated on MDCK cells (25). The SOIV strain A/Regensburg/D6/2009 (H1N1v) was obtained from the Friedrich-Loffler-Institut, Federal Research Institute for Animal Health, Riems, Germany, and grown on MDCK cells.

All animal studies were approved by the Institutional Animal Care and Use Committee of Tuebingen, Germany. For infection of mice, the animals were anesthetized by i.p. injection of 200 µl ketamine (Sanofi-Aventis, Frankfurt, Germany)-rompun (Bayer, Leverkusen, Germany) solution (equal amounts of a 2% rompun solution and a 10% ketamine solution were mixed at the ratio of 1:10 with PBS) and infected intranasally with a 10-fold 50% mouse lethal dose (MLD50) specific for each HPAIV strain ($2 \times 10^7$ PFU for H7N7, $2 \times 10^6$ PFU for H5N1). For both the low pathogen H5N2 and the H1N1v strain, $1 \times 10^4$ PFU (<2MLD50) was used for infection.

Abs and flow cytometry analysis

The following fluorochrome-conjugated Abs were purchased from BD Biosciences (Heidelberg, Germany): mAbs to mouse CD4 (L3T4), CD8a (Ly-2), CD11c (integrin αx chain), CD3 (CD3ε chain), SiglecF, and CD103. Additionally, goat anti-influenza A Ab (AbD Serotec, Dusseldorf, Germany) and donkey anti-goat Alexa Fluor 647 (Invitrogen, Karlruhe, Germany) were used. For flow cytometry analysis, a single-cell suspension was stained in FACS buffer (PBS, 2% FCS, EDTA (pH 7.5)) with required fluorochrome-conjugated Abs for 30 min at 4°C. After the incubation time, cells were washed and analyzed by the FACS Calibur (BD Biosciences).

Quantitative real-time PCR

Total RNA was isolated from the cell homogenate of organs using TRIzol reagent (Invitrogen). A total of 1 µg RNA was used for quantitative real-time RT-PCR (qRT-PCR) to determine the expression of Gapdh, CXCL12, CCL5, ICAM-1, and VCAM using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol, and the SmartCycler System and software (Cepheid, Sunnyvale, CA). The following specific primers for qRT-PCR were used: Mm_Gapdh_2_SG, Mm_Cxcl12_1_SG, Mm_Ccl25_1_SG, Mm_Icam1_1_SG, and Mm_Vcam1_1_SG (Qiagen). The protocol for qRT-PCR was an initial incubation at 50°C for 10 min, and after that, 95°C for 3 min, followed by 40 cycles of 95°C for 10s and 60°C for 30 s. Afterwards, melt curve data were collected from 40–90°C at a ramping rate of 0.2°C/s, and finally, cooling to 40°C was performed. The relative expression values were normalized to the expression value of the housekeeping gene Gapdh.

Detection of viral M-protein RNA was performed as previously described (26) using the following oligonucleotides: 5′-AGAGT AGGA TCTT TTC TAA CCC AOG TCG-3′ (forward), 5′-TGG AAA AAC ATC TTC AAC TGG AAG TCT-3′ (reverse), and 5′-Tet-CTG CCC CTC AAA GCC GA-BHQ-1-3′ (sonde; Metabion International, Martinsried, Germany).

In vitro cytotoxicity assay

Effector cytotoxic CD8+ lymphocyte activity was measured by in vitro cytotoxicity assay. Therefore, five mice were infected with a 10-fold MLD50 of H7N7 or H5N1 virus. Six days postinfection, mice were sacrificed, and leukocytes from the lung and thymus were isolated. Meanwhile, H2B6 MC57 target cells were loaded with the immunodominant peptide nucleoprotein (NP)666-741 in a concentration of 10 µg/ml and labeled with 0.2 mCi [$^3$H]Cr for 37 h for 1 h. For all avian influenza virus strains, the peptide ASENMEAM was used, and for pandemic H1N1, the peptide ASNENVEM was used. Target cells were coinubated with the isolated leukocytes at various E/T ratios in a final volume of 200 µl/well. After 5 h, supernatant was collected and measured for the presence of released [$^3$H]Cr using the microβ counter (Wallac, PerkinElmer, Rodgau, Germany). The percentage of [$^3$H]Cr release was calculated according to the formula previously described (27).

Influenza virus titration (Avicel plaque assay)

The titration in 96-well plates was performed as described previously (25). Briefly, organs from sacrificed mice were homogenized in saline buffer, and target cells were incubated with the homogenate. For H5N2 and H1N1v, trypsin was added during the incubation period (Life Technologies, Rockville, MD). Thereafter, cells were immuno- stained by incubating for 1 h with an mAb specific for the influenza A virus nucleoprotein (AbD Serotec) followed by 30 min incubation with peroxidase-labeled anti-mouse Ab (Dianova, Hamburg, Germany). Labeling was visualized by 10-min incubation with True Blue peroxidase substrate (KPL, Gaithersburg, MD). Stained plates were scanned on a flatbed scanner, and the data were acquired by PhotoStudio5.5 (ArcSoft, Fremont, CA) software. Stained foci were counted. The virus titer is given as the logarithm to the base 10 of the mean value. The detection limit for this test was <1.7 log 10 focus forming units (ffu)/ml.

In situ histology

For in situ hybridization, various organs including lung, thymus, brain, heart, and kidney were removed, fixed overnight in 4% buffered para-
formaldehyde, and embedded in paraffin. Five-micrometer-thick tissue sections were used for in situ stainings.

**In situ hybridization**

Influenza RNA in tissues was detected using single-stranded $^{35}$S-labeled RNA probes that were synthesized from a bluescript KS+ vector containing a fragment of the NP gene (nt 1077 to nt 1442) of A/FPV/Rostock/34 (H7N1) as previously described (28). Control RNA probes were obtained from a transcription vector containing the plasmid of coxsackievirus B3 [pCVB3-R1 (29)]. Pretreatment, hybridization, and washing conditions of dewaxed paraffin tissue sections were performed as described previously (29). Slide preparations were subjected to autoradiography, exposed for 3 wk at 4˚C, and counterstained with H&E.

**Preparation of single-cell suspension for DC isolation**

Isolation of DCs was performed as described previously (30). Mice were sacrificed, and lungs were perfused with 10 ml PBS. Afterward, lungs or thymi were minced and digested in RPMI 1640 medium (Life Technologies) together with 0.125% collagenase II (Roche, Basel, Switzerland) for 30 min at 37˚C in 5% CO$_2$. Thereafter, the minced and digested fractions were centrifuged at 2000 g to obtain cell pellets.

**FIGURE 1.** Reduction of CD4$^+$ T lymphocytes (A) and CD8$^+$ T lymphocytes (B) in the mediastinal LN after 6 d of influenza virus infection. Two independent experiments with four mice per group were performed. Morphology of the thymus after influenza virus infection in mice (C–H). Visual atrophy of the thymus postinfection with high pathogenic H7N7 (C) or H5N1 (D) virus strains but not by low pathogenic H5N2 (E) or pandemic H1N1v (F) influenza virus. All mice were sacrificed 6 d postinfection. Images show uninfected C57BL/6 thymus (top row) in comparison with thymus of infected mice (bottom row). G, Total number of counted leukocytes in the thymus after influenza virus infection. Number of animals: control, n = 8; H7N7, n = 9; H5N1, n = 9; H5N2, n = 4; H1N1v, n = 4. Paired t test revealed significant difference compared with uninfected controls. *p < 0.05; **p < 0.01. H, Changes of the thymocyte populations and single-positive T lymphocytes 6 d after HPAIV influenza virus infection analyzed by flow cytometry. Thymus population after H5N2 and H1N1v are not listed, but show comparable distribution to controls. Four mice per group were used in the experiments. Five independent experiments yielded consistent results.
were passed through a 70-μm BD cell strainer (BD Biosciences) and washed three times. The cell pellets were resuspended in PBS containing 2 mM EDTA and 0.5% BSA for the following DC purification steps.

**Purification of RDCs and DCs**

RDCs were isolated as previously described (30). Briefly, alveolar macrophages were removed by incubating the counted cells with a PE-labeled anti-SiglecF Ab. Thereafter, magnetic microbeads were used for cell separation according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were labeled with a magnetic anti-PE microbead and separated by negative selection using an AutoMACS Separator (Miltenyi Biotec). In the next step, the SiglecF+ fraction was incubated with anti-CD11c magnetic microbeads, and CD11c+ SiglecF− cells were obtained by positive selection according to the manufacturer’s protocol (Miltenyi Biotec). Isolation of thymus DCs was accomplished similar to the isolation steps of RDCs. In contrast to the RDC purification protocol, SiglecF+ macrophages were not depleted in the thymus.

**Adoptive transfer**

Donor C57BL/6-GFP+ mice were infected with the H7N7 influenza virus and sacrificed 3 d postinfection. A total of 4.5 × 106 CD11c+ SiglecF− cells was isolated from the lung of C57BL/6-GFP+ mice and injected intranasally into C57BL/6 wild-type recipient mice. Two days later, C57BL/6 recipient mice were also sacrificed, and both lung and thymus were harvested. Single-cell suspensions were generated, and CD11c+ cells were isolated using magnetic microbeads according to the manufacturer’s protocol (Miltenyi Biotec). Purification was analyzed with an FACSCaliber (BD Biosciences). Cells were surveyed by immunofluorescence.

**Immunofluorescence**

Isolated CD11c+ cells were fixed for 30 min in 4% buffered paraformaldehyde at 4°C. The fixed cells were washed twice with PBS and incubated with DAPI. Afterwards, cells were washed again, mounted with ProLong Gold Antifade Medium (Invitrogen), and analyzed by the ApoTome Care Invert Microscope Labovert FS (Leitz, Wetzlar, Germany) and the AxioscVision Rel 4.5 (Carl Zeiss Imaging, Oberkochen, Germany).

**Immunohistology**

Two-micrometer-thin slides were obtained from paraffin-embedded organs. Sialic acid staining was performed as previously described (31). Briefly, the staining of sialic acid α-2,6 linked to galactose was performed with Sambucus nigra agglutinin, whereas for sialic acid α-2,3 linked to galactose Maackia amurensis, agglutinin was used (Vector Laboratories, Burlingame, CA). Signal detection was visualized by an ABC and DAB kit according to the manufacturer’s protocol (Vector Laboratories). For viral detection, slides were incubated with the goat anti-influenza A Ab (AbD Serotec), and the ImmPRESS REAGENT Anti-Goat Ig Kit (Vector Laboratories) was used for secondary staining. Substrate reaction was accomplished with a DAB kit (Vector Laboratories), and counterstaining was performed with hematoxylin Gill II (Carl Roth, Karlsruhe, Germany). The staining was evaluated by microscope (Leitz) and photographed with the DC 300 (Leica Solms, Germany).

**Statistical analysis**

Error bars are given as the SEM. For the investigation of significant differences (p < 0.05), the paired t test was performed.

**Results**

*H5N1 influenza virus interferes with T lymphocyte development*

To investigate the mechanism of influenza virus-mediated lymphopenia, mice were infected for 6 d with either H7N7 or H5N1, two highly pathogenic avian influenza virus strains, the pandemic H1N1v human influenza strain, or a low pathogenic H5N2 virus of avian origin. Thereafter, the animals were sacrificed. CD4+ and CD8+ T lymphocytes in the mediastinal lymph node (LN) were quantified by flow cytometry (Fig. 1A, 1B). Both HPAIV strains revealed a reduction, whereas the low pathogenic avian influenza virus strain and the human H1N1 strain showed no significant changes in the amount of CD4+ and CD8+ T lymphocytes. A reduction of the T lymphocyte population after HPAIV infection was additionally observed in the blood (data not shown). Also, the morphology of the thymus was analyzed. During infection with either H7N7 or H5N1 virus, the thymus underwent a strong atrophic process that led to a drastically reduced size already 6 d after virus infection (Fig. 1C, 1D, bottom row), which was not found after H1N1v or H5N2 influenza virus infection (Fig. 1E, 1F, bottom row). H7N7 infection led to an 84% reduction of the total leukocyte count (Fig. 1G, white bars). After H5N1 infection, the total leukocyte amount was even more drastically reduced to <5% compared with total leukocytes of the thymus of uninfected mice (Fig. 1G, vertical line bar). No significant leukocyte depletion was observed after H5N2 or H1N1v infection (Fig. 1G, gray and horizontal line bar). Analysis of cells within the thymus revealed that especially the CD4+CD8+ thymocyte population was nearly absent after HPAIV infection. However, CD4+CD8+ thymocytes of control, H5N2-, and H1N1v-infected mice presented ~80–85% of the cells in the thymus and extinguished ~1 × 106 cells. This population was reduced to 5% and dropped to ~1 × 105 thy-
mocytes after H7N7 but also H5N1 infection (Fig. 1H, Supplemental Fig. 1). These results suggested that HPAIV infection of mice interferes with T lymphocyte development or thymocyte progenitor cells. Because the bone marrow is the source for thymocyte progenitor cells, we next investigated whether HPAIV is present in the bone marrow using qRT-PCR to detect viral RNA. However, no viral nucleic acid could be found in the bone marrow in mice 3 and 6 d postinfection with either H7N7 or H5N1 (data not shown). These results indicated that the bone marrow itself is not targeted by HPAIV influenza virus infection. To further investigate the influence of influenza virus infection of the thymus, expression of chemokines (CXCL12, CCL25) and cell adhesion molecules (ICAM-1, VCAM-1) that are involved in T lymphocyte development was analyzed. Infection of mice with H7N7 led to a strong downregulation of mRNA encoding for CXCL12 and CCL25 that is produced by thymic epithelial cells in the cortex and the medulla (Fig. 2A). The mRNA encoding for either CXCL12 or CCL25 was less reduced after H5N1 infection. In contrast, ICAM-1 mRNA expression at day 6 was even slightly increased (Fig. 2B). No strong alterations of the thymus or thymocyte development were found after H5N2 or H1N1v influenza virus infection (Fig. 2C,2D). These results indicate that the thymus T lymphocyte development is altered during an HPAIV infection and give rise to the assumption that the function of thymic epithelial cells is impaired. Thus, HPAIV infection affects the thymus and could interfere with the thymic homeostasis.

Presence of influenza virus-specific CD8+ T cells in the thymus after HPAIV infection

The previous results allowed the idea that the altered function of the thymic epithelial cells might have an influence on the impaired T lymphocyte development in the thymus after HPAIV infection, including the dramatic loss of CD4+CD8+ thymocytes. Therefore, we next questioned whether the activation status of the immune lymphocytes in the thymus was altered after influenza virus infection. Whereas in the thymus of uninfected mice, the amount of activated thymocytes is <5% within this population, the number increased up to ~50% after H5N1 or H7N7 infection. No significant change of the thymocyte activation was observed after H5N1 or H1N1v influenza virus infection. However, HPAIV infection also changed the activation status based on CD69 expression of the single-positive lymphocyte populations in the thymus. Although there was no strong increase of the activated CD4+ T lymphocyte population, the activated CD8+ T lymphocyte fraction rose from 23% of uninfected mice up to 47% of H5N1-infected mice (Supplemental Fig 2). To determine whether activated CD8+ T lymphocytes in the thymus were specific for influenza virus, an in vitro cytotoxicity assay was performed using target cells labeled with the immunodominant peptide NP366–377 derived from the viral nucleoprotein (Fig. 3). As a control, influenza virus-specific CD8+ T lymphocytes could be detected in the lungs of mice after H5N1, H7N7, H1N1v, or H5N2 influenza virus infection (Fig. 3, black solid line). Most surprisingly, after

FIGURE 3. In vitro cytotoxicity assay of influenza virus-specific CD8+ lymphocytes. 51Cr-labeled MC57 target cells were loaded with the immunodominant influenza peptide NP366–374. NP specificity of CD8+ T lymphocytes isolated from lung and thymus 6 d postinfection with H7N7(A), H5N1 (B), H5N2 (C), or H1N1v (D) were determined by the [51Cr] release of lysed target cells. Two experiments were performed independently with three mice.

FIGURE 4. Immunohistological detection of sialic acids in the lung, mediastinal LNs, and thymus of C57BL/6 mice (A) and the mesenterial LN and thymus of humans (B). Avian influenza viruses recognize Siaα2-3gal, whereas human influenza viruses use Siaα2-6gal as a receptor for infection. Original magnification ×20. For each tissue, at least five slides were analyzed.
H7N7 and H5N1 infection of mice, nucleoprotein-specific CD8+ T lymphocytes were also found in the thymus (Fig. 3A, 3B, gray solid line). In contrast, no NP366–377-specific CD8+ T cells were notable in the thymus after H5N2 or H1N1v infection (Fig. 3C, 3D, gray solid line). These results demonstrated that postinfection with both HPAIV strains, functional influenza virus-specific cytotoxic CD8+ T lymphocytes were present in the thymus to recognize infected target cells in an MHC class I-restricted manner.

Infectious H5N1 and H7N7 influenza viruses are present in the thymus

The above results indicate a direct interaction of H5N1 and H7N7 influenza virus with the thymus. Avian influenza viruses require Siaα2-3gal as a receptor. Siaα2-6gal is a prerequisite for infection with human influenza viruses. To determine whether the thymus of mice and humans are sensible for avian or human influenza virus infection, immunohistology was performed to scrutinize the distribution of Siaα2-3gal and Siaα2-6gal. In addition to lung and mediastinal LNs, Siaα2-3gal and Siaα2-6gal expression was found in the thymus of both species, whereas especially the medulla region showed positive signals for Siaα2-3gal and Siaα2-6gal (Fig. 4). To analyze whether infectious influenza virus is present in the thymus of mice, virus infectivity assays were performed. H7N7 and H5N1 influenza virus was found to high titers in the lung and to moderate titers in the mediastinal LNs and in the thymus. In addition, virus was present in the spleen after H7N7 but not after H5N1 infection. In the lung of H1N1v-infected mice, comparable titers to H5N1- and H7N7-infected mice were found. In contrast, only low amounts of infectious virus were present in the mediastinal LNs. In only one out of four thymi, few infectious virus particles could be detected. After H5N2 influenza virus infection, low amounts of virus were found in the lung, and only one out of four mice revealed some infectious particles in the medullary LNs. No virus was found in the thymus (Table I). qRT-PCR was used to confirm the data achieved by virus infectivity assay after HPAIV infection and to observe the progression of viral load in the lymphoid organs (Fig. 5A). The results demonstrate viral nucleic acid in the thymus of mice postinfection with the HPAIV virus strains. In addition, viral RNA was imaged by in situ hybridization in the lung and thymus (Fig. 5B). The in situ hybridization experiments confirm the existence of viral footprints in both organs after H5N1 and H7N7 influenza virus infection and localized the infectivity in the thymus to the corticomedullary junctions. By imaging the regions of the thymus that are virus positive, the question appears to be

### Table I. Distribution of the viral load 6 d postinfection of C57BL/6 mice

<table>
<thead>
<tr>
<th></th>
<th>Lung</th>
<th>Mediastinal LN</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7N7</td>
<td>5.24 ± 0.19</td>
<td>3.71 ± 0.24</td>
<td>3.85 ± 0.23</td>
<td>3.61 ± 0.22</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>H5N1</td>
<td>5.29 ± 0.06</td>
<td>3.80 ± 0.35</td>
<td>2.84 ± 0.21</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>H5N2</td>
<td>2.91 ± 0.52</td>
<td>2.05 ± 0.31a</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>H1N1</td>
<td>5.65 ± 0.49</td>
<td>2.22 ± 0.77</td>
<td>1.82 ± 0.10a</td>
<td>3.27 ± 0.28</td>
<td>&lt;1.7</td>
</tr>
</tbody>
</table>

Virus titers are given as the logarithm in focus forming units per 1 ml organ homogenate.

*Low amounts of virus were detected in one out of four organs (H5N2 mediastinal LN: 3.12 ffu/ml; H1N1 thymus: 2.01 ffu/ml).
which route of infection the virus uses to reach these specific thymus cells.

Infection of lung DCs by H5N1, H7N7, and human pandemic H1N1v influenza virus

Circulating DCs, in addition to mature peripheral T lymphocytes, can re-enter the thymus after the uptake of antigenic material in the periphery (16). Next to this DC attribute, it was demonstrated that DCs are a target for influenza virus infection (30). To investigate whether DCs harbor influenza virus, mice were sacrificed, and RDCs were isolated 3 d postinfection. DCs were identified by CD11c+ and SiglecF+ staining. CD11c is found on DCs and macrophages. SiglecF, a sialic acid binding Ig-like lectin, is expressed by macrophages but not by DCs. Flow cytometry analysis demonstrated a high infection rate of DCs either after H5N1 or H7N7 influenza virus and, to a lower extent, after pandemic H1N1v infection (Fig. 6). H5N1 virus infected ∼19.0% of DCs in the lung, whereas 13.5% of DCs were infected with H7N7 influenza virus. Only 9.2% DCs were infected with the H1N1v virus strain. In contrast, after H5N2 influenza virus infection of mice, no CD11c+ cells harbored viral footprints. To define the DC subpopulation being the target for influenza virus in more detail, DCs were stained against CD103, which is a marker for mucosal DCs (Fig. 6A, middle row). The flow cytometry analysis demonstrated that the CD11c+/CD103+/SiglecF+ DC population is a preferred target for influenza virus. Nevertheless, CD11c+/CD103+/SiglecF− DCs present roughly only 13% of the DC population (data not shown). Our results indicate that macrophages are not the obvious main target for influenza virus infection. These findings suggest a potential role of migrating DCs in the viral spread to the thymus.

To investigate whether lung DCs migrate to the thymus postinfection with HPAIV, DCs from H7N7-infected C57BL/6-GFP+ mice were isolated and transferred into the lungs of GFP+ mice (Fig. 7A). Three days after the transfer, recipient mice were sacrificed, and GFP+ DCs were isolated from the lung and thymus. Isolated DCs were characterized by immunofluorescence. The analysis clearly demonstrated that GFP+CD11c+ cells were detectable in lung and, most interesting, in the thymus of recipient wild-type mice, providing evidence that respiratory DCs migrate to the thymus postinfection with HPAIV (Fig. 7B). These results strongly propose a model by which HPAIV hitchhike DCs in the lung for trafficking into the thymus, leading to alterations of the Ag presentation for developing thymocytes (Fig. 8).

Discussion

A common feature of human infection with pandemic avian influenza virus is a strong reduction of T lymphocytes. This feature, called lymphopenia, is also found in some other infectious diseases, in particular with new emerging pathogens (8, 9). Nevertheless, the reason and mechanism for the declining number of lymphocytes remain unclear (32). To investigate a possible mechanism for lymphopenia after influenza virus infection, we focused on the thymus as a primary lymphoid organ. We hypothesized that highly pathogenic influenza A virus infection of mice interferes with
T lymphocyte development and selection processes in the thymus, which consequently leads to an impaired cellular immune response. The hypothesis was developed because the morphology of the thymus revealed massive destruction after H5N2 and H7N7 influenza virus infection of mice but not after pandemic H1N1v and low pathogenic H5N2 influenza virus infection. Therefore, reduced or even missing naive T lymphocytes outflow could lead to the observed lymphopenia. We speculate a direct involvement of the highly pathogenic avian influenza A virus. Two reasons for such a dysregulation followed by the destruction of the T lymphocyte-producing site might be possible. First, the virus stops the influx of T lymphocyte progenitor cells directly by infecting the bone marrow, which is the source of T cell progenitors. Second, influenza virus infection interferes with T lymphocyte development directly in the thymus. Because qRT-PCR experiments revealed no traces of viral RNA in the bone marrow, we can exclude that the observed phenomenon is due to infection of the bone marrow. The hypothesis that HPAIV interferes with T lymphocyte development was supported by the findings that the expression of chemokines (CXCL12, CCL25) and cell adhesion molecules (ICAM-1, VCAM-1) involved in T cell development was drastically reduced after HPAIV but only slightly after H1N1v or H5N2 influenza virus infection. CXCL12, which is recognized by the CXCR4 receptor, is an essential prerequisite for functional thymocyte migration. CXCL12 is produced by both cortical and medullar epithelial cells, and recent studies underline the urgent need for CXCL12/CXCR4 pathway in T lymphocyte development in vivo. Adoptive transfer of GFP+ DCs into H7N7 (10-fold MLD_{50}) influenza virus-infected wild-type mice. A, Experimental design: DCs from infected C57BL/6-GFP-expressing mice were isolated 3 d postinfection and transferred into lungs of wild-type C57BL/6 mice. Recipient mice were sacrificed 2 d posttransfer, and GFP+ DCs found in the lung and thymus (<1%) were visualized by immunofluorescence (B). Experiment was performed twice with groups of two mice. After H1N1v infection, virus was found in the thymus of one mouse in a low amount. This result gives rise to the assumption that this new emerging human pandemic influenza virus strain has the ability to infect the thymus. Nevertheless, from our data, we speculate that a so far unknown viral piece is still absent in this recent pandemic H1N1v strain to induce fatal disease. This observation could underline the fact that pandemic H1N1v has a high morbidity coming with a mild illness, whereas fatal cases are fairly rare.

FIGURE 7. Adoptive transfer of GFP+ DCs into H7N7 (10-fold MLD_{50}) influenza virus-infected wild-type mice. A, Experimental design: DCs from infected C57BL/6-GFP-expressing mice were isolated 3 d postinfection and transferred into lungs of wild-type C57BL/6 mice. Recipient mice were sacrificed 2 d posttransfer, and GFP+ DCs found in the lung and thymus (<1%) were visualized by immunofluorescence (B). Experiment was performed twice with groups of two mice.
macrophages were not the major target for infection. More interestingly, it has been reported that DCs can be infected by influenza viruses in vitro (30). Because of the DC function to migrate continuously into the LN but also in the thymus (15) with or without having contact and being activated by an Ag, thymic DCs play a major role in establishing tolerance by negative selection of thymocytes and induction of Tregs (45). We hypothesized that DCs are the carrier cell type for influenza viruses. We first tested whether different influenza virus strains were able to infect DCs in the lung. Flow cytometry analysis demonstrated a high rate of DC infection with the HPAIV strains and to a lesser extent with the H1N1v strain. Low pathogenic H5N2 virus infection of DCs was not detectable. Our results demonstrate that the infection of DCs with HPAIV also includes the CD103+ DC subpopulation, for which an induction of peripheral Tregs has already been described (46). Because CD103+ mucosal DCs only present 13% (data not shown) of the total DC population, we cannot exclude that the virus strains used in this study are specialized on CD11c+/CD103+ subpopulations.

The adoptive transfer experiment using GFP+ lung DCs further strengthens our hypothesis that DCs can migrate from the lung into the thymus. Flow cytometry and immunofluorescence revealed the presence of GFP+ cells in the thymus of HPAIV-infected wild-type mice. Nevertheless, from our immunofluorescence data, we cannot exclude that these respiratory DCs in the thymus undergo apoptotic processes due to either influenza virus infection or CD8+ T cell response. From these results, we hypothesize that activated, HPAIV-infected DCs move from the lung into local, mediastinal LNs, where viral replication can take place. In a second step, DCs move further on into the thymus, where influenza virus-infected DCs first reach the corticomedullary junction and the medulla, the place of negative selection processes. Thymic APCs in the thymus, namely DCs, but also thymic epithelial cells, are responsible for the selection processes (47). We conclude that CD45− epithelial cells in the medulla are the first target for infection because viral footprints in the CD45+ thymocyte population were absent (data not shown). Epithelial cells are critical for the development of T lymphocytes. These stroma cells give the structure for a working positive and negative selection of outgoing naive T lymphocytes. An interference with CD45− cells can lead to a dysregulation of T lymphocyte development, especially in the younger population when the thymus is still highly active. One result could be a reduced or even missing immune response against new Ags that break through the mechanical barriers of our immune system. The pandemic H1N1v strain does not reach the thymus consistently, which could be due to a missing pathogenesis factor, because virus titer is even higher in the lung of H1N1v-infected mice compared with viral titers in the lung after H5N1 and H7N7 infection.

We propose a mechanism in which highly pathogenic avian influenza virus has the ability to infect DCs in the lung. This migrating cell type can now transport the virus to secondary lymphoid organs like LNs, but also, more importantly, to the primary lymphoid organ, the thymus. In both cases, the infected cell can not only support the virus to spread to selected targets but also present viral Ags as pseudo self-peptides. That way, an additional clonal deletion of influenza-specific T lymphocytes can take place in the thymus. The collapse of the thymocyte’s developing signal network could be because stromal cells in the thymus are a target for the virus (Fig. 8). Additionally, influenza-specific Tregs could be generated that suppress already existing T lymphocyte activity against viral epitopes. The existence of Tregs needs to be analyzed in further studies to assure this assumption.

Taken together, the infection of DCs demonstrates a very effective way to evade the immune system and could be the reason for fatal influenza virus infection that leads to lymphopenia and the strong influx of innate immune cells (17). The fact that the Siaα2-3gal and Siaα2-6gal lectins that function as the receptor for in-
fluenza virus infection are also expressed in human thymus (Fig. 4) makes the transfer of the described mouse model to the pathogenetic situation in humans likely. The recent pandemic H1N1v outbreak that also causes lymphopenia in severe clinical cases demonstrates that this described mechanism might not only be restricted to highly pathogenic avian influenza viruses, but might be a general feature of severe influenza (48).

Acknowledgments
We thank Carmen Mueller, Isabell Laurisch, Mirijam Steck, Ulrich Wulle, and Martina Sauter for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Calculated number of (A) CD4+ or (B) CD8+ T lymphocytes and (C) DP thymocytes 3 or 6 days after influenza virus infection. The quantity was evaluated by the total number of leukocytes divided by the gated quotient of the flow cytometry analysis as shown in Fig. 1. Paired t-test revealed significant difference compared to control (*p < 0.05; **p < 0.01). Five independent experiments yielded consistent results.
**Supplementary Figure 2.** Investigation of CD69$^+$ activated (A) CD4$^+$ or (B) CD8$^+$ T lymphocytes and (C) DP thymocytes in the thymus. The analysis shows an increase of activated CD8$^+$ lymphocyte after H5N1 infection and a strong boost of activated DP thymocytes after HPAIV infection on day 6 p.i. Paired t-test revealed significant differences compared to control (*$p < 0.05$; **$p < 0.01$). 3 mice were tested in three different experiments.