Unraveling the role of B cells in the pathogenesis of an oncogenic avian herpesvirus

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Marek’s disease virus (MDV) is a highly oncogenic herpesvirus that causes immunosuppression, paralysis, and deadly lymphomas in chickens. In infected animals, B cells are efficiently infected and are thought to amplify the virus and transfer it to T cells. MDV subsequently establishes latency in T cells and transforms CD4+ T cells, resulting in fatal lymphomas. Despite many years of research, the exact role of the different B and T cell subsets in MDV pathogenesis remains poorly understood, mostly due to the lack of reverse genetics in chickens. Recently, Ig heavy chain J gene segment knockout (JH-KO) chickens lacking mature and peripheral B cells have been generated. To determine the role of these B cells in MDV pathogenesis, we infected JH-KO chickens with the very virulent MDV RB18 strain. Surprisingly, viral load in the blood of infected animals was not altered in the absence of B cells. More importantly, disease and tumor incidence in JH-KO chickens was comparable to wild-type animals, suggesting that both mature and peripheral B cells are dispensable for MDV pathogenesis. Intriguingly, MDV efficiently replicated in the bursa of Fabricius in JH-KO animals, while spread of the virus to the spleen and thymus was delayed. In the absence of B cells, MDV readily infected CD4+ and CD8+ T cells, allowing efficient virus replication in the lymphoid organs and transformation of T cells. Taken together, our data change the dogma of the central role of B cells and thereby provide important insights into MDV pathogenesis.

Marek’s disease virus | B cells | Lymphomagenesis | Ig knockout chickens | Transgenic chickens

Significance

Marek’s disease virus (MDV) infects chickens and causes the most frequent clinically diagnosed cancer in the animal kingdom, and it is used as a small-animal model for virus-induced tumor formation. Until now, B cells were thought to play a central role in MDV pathogenesis. We disproved this dogma using knockout (KO) chickens that lack mature and peripheral B cells. We demonstrated that B cells are dispensable for virus replication, virus spread, and tumor formation. In the absence of B cells, T cells facilitate efficient virus replication and are subsequently transformed, leading to fatal lymphomas. Our data pioneer the use of KO chickens in infectious disease research and expand our knowledge on the life cycle of this highly oncogenic virus.
described in mammalian species (22–25), resulting in the abrogation of B cell maturation and migration out of the bursa. To study the role of B cells in MDV pathogenesis, we infected wild-type (wt), heterozygous (JH+/−), and homozygous (JH−/−) JH-KO birds. Surprisingly, we could demonstrate that mature and peripheral B cells are completely dispensable for MDV pathogenesis. In the absence of B cells, MDV efficiently replicated in birds. Surprisingly, we could demonstrate that mature and peripheral B cells are completely dispensable for MDV pathogenesis. In the absence of B cells, MDV efficiently replicated in birds. Surprisingly, we could demonstrate that mature and peripheral B cells are completely dispensable for MDV pathogenesis.

Results

Infection and Confirmation of B Cell KO Chickens. To determine the role of B cells in MDV pathogenesis and tumor formation, we used transgenic chickens that lack mature and peripheral B cells (JH-KO). The genotype of all animals was determined posthatching by PCR assays as described previously (21). We subsequently infected 1-d-old wt (n = 17), JH+/− (n = 22), and JH−/− (n = 21) chickens intraabdominally with the very virulent RB-1B MDV strain. To confirm that the JH−/− chickens indeed lack B cells, we assessed the presence of antibodies in the blood by ELISA 28 d postinfection (dpi) as described previously (21). We demonstrated that IgM antibodies were completely absent in JH−/− animals and comparable to the PBS control (Fig. L4), suggesting that no mature and peripheral B cells were present. In addition, IgY titers in JH−/− chickens were highly reduced compared with wt and JH+/− chickens, indicating that only residual maternal antibodies are present at day 28 (Fig. 1B). Beyond that, we confirmed the absence of B cells in the spleen of JH−/− chickens by immunohistochemistry using the B cell marker AV20 (Fig. 1C).

MDV Replication and Tumor Formation Are Not Altered in the Absence of B Cells. To examine if MDV replication is altered in the absence of B cells, we analyzed MDV genome copy numbers in peripheral blood by qPCR. Surprisingly, MDV could efficiently replicate in the absence of B cells in the infected animals (Fig. 2A), suggesting that B cells are not required for the amplification of the virus during lytic replication. Furthermore, disease incidence was not altered in chickens that lack B cells compared with their hatch mates (Fig. 2B), indicating that B cells are not required for the development of Marek’s disease. Beyond that, no significant difference in tumor incidence was observed compared with wt and JH+/− animals until 13 wk postinfection (Fig. 2C), suggesting that B cells are also dispensable for the transformation process and lymphomagenesis. Furthermore, the dissemination of tumors was not altered in the absence of B cells (Fig. 2D). These data demonstrate that mature and peripheral B cells are dispensable for virus replication in vivo, disease development, tumor formation, and dissemination.

B Cells Are also Dispensable in the Natural Infection of MDV. To confirm that mature and peripheral B cells are also not required during natural infection via the respiratory route, we housed naive contact chickens together with the intraabdominally infected animals. MDV spread to naive individuals that do not have B cells, indicating that B cells in the lung are not essential for early infection via the natural route. As observed in the experimentally infected animals, disease and tumor incidence was also not altered in naturally infected animals in the absence of B cells (Fig. 3). Taken together, our data confirm that mature and peripheral B cells are dispensable for MDV pathogenesis during natural infection.

MDV Efficiently Spreads to Lymphoid Organs in the Absence of B Cells. To determine if B cells play a role in the initial spread of the virus to the lymphoid organs during early lytic infection (26), we investigated the viral load in the major lymphoid organs in chickens with (JH+/−) and without (JH−/−) B cells at 4, 7, 10, and 14 dpi by qPCR. Intriguingly, MDV efficiently spread to the
Intriguingly, very few immature B cells were infected in the bursa and spleen. Our data show that mature and peripheral B cells are dispensable in the absence of B cells once it reaches thymus, bursa, and spleen. These findings indicate that T cells compensate for the loss of peripheral and mature B cells. In the absence of B cells, viral load in the thymus was significantly reduced at day 4 post-infection (Fig. 4B) and severely reduced in the spleen at day 4 post-infection (Fig. 4C). Together, these data indicate that B cells may support early dissemination of MDV into the lymphoid organs. However, similar viral levels were reached between 7 and 14 dpi, suggesting that the virus can efficiently replicate in the absence of B cells once it reaches thymus, bursa, and spleen. Our data show that mature and peripheral B cells are dispensable for the spread of MDV within the infected host.

MDV Lyratically Infects CD4+ and CD8+ T Cells in the Absence of B Cells.

To identify the cell types infected in the absence of peripheral and mature B cells, we performed immunohistochemistry on the major lymphoid organs at 7 dpi and quantified the infected target cells. In the presence of B cells, MDV predominantly infected B cells in the spleen (Fig. 5B) as described previously (27). In contrast, MDV almost exclusively infected CD4+ and CD8+ T cells in the spleen in the absence of B cells (Fig. 5B). Similarly, CD4+ and CD8+ T cells were the main target in the thymus and bursa of infected JH−/− chickens (SI Appendix, Fig. S1A and B). Intriguingly, very few immature B cells were infected in the bursa of JH−/− chickens, suggesting that these cells are not very permissive to infection. Our data demonstrate that infection of the target cells for latency and transformation (CD4+ and CD8+ T cells) compensates for the loss of peripheral and mature B cells, resulting in high viral titers in the major lymphoid organs, disease progression, and tumorigenesis.

Discussion

Until now, B cells were thought to be the primary target cells for MDV lytic replication and responsible for virus amplification in susceptible hosts (reviewed in refs. 1, 28). This was mostly based on the observation that B cells are efficiently infected in vitro and in vivo. Others previously set out to address the role of B cells and the bursa in MDV pathogenesis by either chemical depletion of B cells and/or surgical removal of the bursa of Fabricius as the site of B cell development. Unfortunately, these studies did not provide a clear answer to the role of B cells as disease and tumor incidence in these animals was increased (20), was decreased (13–17), or did not show any difference compared with the controls (18, 19). These divergent results could have been caused by off-target effects of the drugs, treatments, and degree of B cell depletion. For example, drug treatment can affect other lymphocyte populations such as T cells, the main target cell for establishment of latency and transformation, and can result in incomplete removal of B cells. Similarly, a partial resection of the bursa would only result in a reduced level of B cells. Furthermore, removal of the bursa not only affects the development of B cells but also removes the pool of immature progenitor lymphocytes of the bursa, as discussed further below. Unfortunately, until recently, there was no KO technology available in chickens to address this long-standing question.

Schusser et al. (21) recently generated and extensively characterized KO chickens in which the JH was deleted. This deletion abrogates B cell maturation and antibody production in these chickens, as shown above (Fig. 1 A, B). Also, no B cells were detected in tissue samples of JH−/− KO chickens by immunohistochemistry (Fig. 1C). Immature progenitor lymphocytes still migrate into the bursa, but do not leave this lymphoid organ (21). To elucidate the role of mature and peripheral B cells, we infected these chickens with the very virulent RB-1B MDV strain. Surprisingly, virus load in the blood was not altered in the absence of the B cells in these animals compared with their wt and JH+/− siblings (Fig. 2A). This indicates that either B cells do not significantly contribute to the viral load in the blood or other cells can compensate for the loss of peripheral B cells. In addition, disease and tumor incidence was not altered in experimentally infected animals that lack B cells (Fig. 2B and C). This scenario was also observed in animals that were infected via the natural route of infection (Fig. 3).

In our experiments, we also found that JH-KO chickens and their wt siblings are relatively resistant to MDV infection. Viral load in the blood as well as disease and tumor incidence were
similar to resistant chicken lines [e.g., N2a (29, 30)]. However, our animals were less susceptible than other chicken lines frequently used in MDV research. This is likely due to the outbred nature of the JH-KO chickens, which tend to be more resistant to MDV.

A prerequisite for disease and tumor formation is efficient virus replication in the host. To assess the early events in the lytic phase of MDV replication, we analyzed the key lymphoid organs: the bursa, thymus, and spleen. We observed that within 4 d, MDV spreads to the major lymphoid organs even in the absence of peripheral B cells. Although viral load in the thymus and spleen was reduced in the first 4 dpi, the trend of viral replication in the primary lymphoid organs was similar in chickens lacking mature B cells and their normal siblings (Fig. 4A), indicating that other cells can compensate for the loss of B cells. The delay in the spread of the virus to the thymus and spleen suggests that B cells only play a minor role in the early dissemination of the virus. Although B cells were thought to be the main target population of MDV replication in wt animals, other targets have been identified. For example, it has been shown that MDV can productively replicate in phagocytes such as macrophages (31, 32) and T cells (27, 33, 34) in vitro. Our data revealed that MDV replicates in the primary target cells for latency and transformation, CD4+ and CD8+ T cells, in the absence of B cells in vivo. Similarly, immature lymphocyte progenitor cells were infected in the bursa, however, to a much lesser extent than mature B cells in wt animals.

In summary, we addressed the role of mature and peripheral B cells in the pathogenesis of MDV, an important veterinary pathogen that is also frequently used as an animal model for herpesvirus-induced tumor formation. Our data surprisingly demonstrate that B cells are dispensable targets of MDV. Within 4 dpi, the virus reaches the bursa, thymus, and spleen independent of B cells. Once MDV reaches these major lymphoid organs, the virus is rapidly amplified in both wt and JH-KO chickens. In the absence of B cells, the target cells of latency and transformation (CD4+ and CD8+ T cells) are directly infected and compensated for the loss of B cells during lytic replication. These data pioneer the use of KO technology to study infectious disease in chickens. In addition, they unequivocally refute the current dogma of the crucial role of B cells in MDV pathogenesis and refocus future studies onto other target cells infected by the virus.

### Materials and Methods

#### Ethics Statement
All animal work was conducted according to relevant national and international guidelines for humane use of animals. Animal experiments were approved by the Landesamt für Gesundheit und Soziales in Berlin (approval no. G 0218/12).

#### In Vivo Experiments
One-day-old MDV maternal antibody-positive wt, JH+/−, and JH−/− chickens (21) were infected intraabdominally with 4,000 plaque-forming units of the very virulent RB-18 clone (GenBank EF523390.1) (35). RB18 stocks were propagated in passaged chicken embryo cells that were generated from Valo specific-pathogen-free embryos (Lohman Tierzucht) and maintained in MEM supplemented with 10% FBS (PAN-Biotech) and penicillin/streptomycin (Applichem) at 37 °C and 5% CO2. Heterozygotes (JH+−) still develop mature and peripheral B cells and produce antibodies comparable to wt birds. Naive chickens were housed with infected animals to assess transmission via the natural route of infection. Water and food were provided ad libitum. Blood samples were taken from infected animals

### Table 1. Primers and probes for genotyping and qPCR

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Direction</th>
<th>Sequence (5′ → 3′)</th>
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<tbody>
<tr>
<td>wt region</td>
<td>For</td>
<td>ATGGGCCAAGGAGGCCAA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GCCCAATATGGCCCAAAC</td>
</tr>
<tr>
<td>JH-KO region</td>
<td>For</td>
<td>AGTGAAAAATTCGCCAAG</td>
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<tr>
<td></td>
<td>Rev</td>
<td>GCCCAATATGGCCAAAC</td>
</tr>
<tr>
<td>ICP4 (qPCR)</td>
<td>For</td>
<td>CGGTCTTCCGCGATGGA</td>
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<td></td>
<td>Rev</td>
<td>TCCCTAACCACATATCTAA</td>
</tr>
<tr>
<td>Probe</td>
<td></td>
<td>FAM-CCCCCAAGGGTCAGTGCAG- TAM</td>
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<tr>
<td>Inducible nitric oxide synthase (qPCR)</td>
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<tr>
<td></td>
<td>Rev</td>
<td>TCCGAGCCTCCCGACCTCA</td>
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<tr>
<td>Probe</td>
<td></td>
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For, forward; Rev, reverse.
at 4, 7, 10, 14, 21, and 28 dpi. Chickens were monitored for clinical symptoms of Marek’s disease on a daily basis throughout the 91-d experiment. To eliminate bias, the examining veterinarian had no knowledge of the genotypes of the birds throughout the experiment. Animals were euthanized and examined for tumor lesions either once clinical symptoms were evident or after termination of the experiment.

Genotyping. DNA was isolated from the blood of chickens using the E-296 96-well blood DNA isolation kit (Omega Biotek) according to the manufacturer’s instructions. PCR primers specific for the J gene segment deletion and the wt locus were used as described previously (21) (Table 1).

ELISA. Total plasma IgM and IgY were measured as described previously (21). Briefly, enzyme immunoassay plates (Sarstedt) were coated with 2 μg/ml goat anti-chicken IgM (Biomol) or goat anti-chicken IgY (Biomol) overnight. Plates were blocked with 4% (wt/vol) skim milk and incubated with chicken plasma for 2 h. Bound chicken IgM or IgY was detected with goat anti-chicken IgM-HRP (Biomol) or goat anti-chicken IgY-HRP (Biomol) and developed with 3,3',5,5'-tetramethylbenzidine substrate. The OD at 450 nm was measured. All samples were measured in duplicate.

Quantification of MDV Genome Copies. DNA was isolated from the blood as described above. In addition, DNA was isolated from the bursa, thymus, and spleen of infected animals using the innuSPEED Tissue DNA Kit (Analytik Jena) according to the manufacturer’s instructions. MDV genome copies were determined by qPCR using specific primers and a probe for the gene encoding MDV ICMP. ICMP copy numbers were normalized against cellular inducible nitric oxide synthase as described previously (36) (Table 1).

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Immunohistochemistry. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C. Before sectioning, samples were embedded in Tissue-Tek (Sakura Finetek). Cryostat sections (5 μm thick) were mounted on Super FrostPlus slides (Thermo Fisher Scientific), air-dried, and fixed in 4% paraformaldehyde for 15 min. Sections were rehydrated in PBS and blocked for 1 h with 1% BSA and 2.5% normal goat serum (Dianova) in PBS. This was followed by a 1-h incubation at room temperature (RT) with a mixture of anti-VP5 (clone F19, 1:3,000) and anti-ICP4 (clone E21, 1:1,000) kindly provided by Jean-François Vautheron (INRA Centre Val de Loire, Nouzilly, France), followed by an overnight incubation at 4 °C with goat-anti-mouse IgG1-AlexaFluor555 (1:500; SBA) and blocking for 1 h with normal mouse serum (5% in PBS). Finally, the cell surface markers anti-CDA-AlexaFluor467 (clone CT4, 1:100; SBA) and anti-Bu1-AF647 (clone AV20, 1:200; SBA) were added for 1 h at RT. For anti-CD8 staining, anti-CD8 (clone 3-298 (37), supernatant 1:50) was added simultaneously with anti-MDV antibodies and anti-mouse IgG2b-APC (1:400; Jackson Immunoresearch Europe) followed by anti-mouse IgG1-AlexaFluor555.

Sections were counterstained with DAPI (100 ng/mL; Sigma-Aldrich) for 10 min and mounted in Prolong Glass Antifade Mountant (Thermo Fisher Scientific). Images were taken using a VisiScope spinning disk confocal microscope (Visitron Systems GmbH). Images were processed using ImageJ software v1.51 (NIH). At least 100 infected cells per organ were evaluated.

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