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Role of intrabursal T cells in infectious bursal disease virus (IBDV) infection: T cells promote viral clearance but delay follicular recovery

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Summary. Infectious bursal disease virus (IBDV) induces an acute, highly contagious immunosuppressive disease in young chickens. We examined the role of T cells in IBDV-induced immunopathogenesis and tissue recovery. T cell-intact chickens and birds compromised in their T cell function by a combination of surgical thymectomy and Cyclosporin A treatment (Tx-CsA) were infected with an intermediate vaccine strain of IBDV (Bursine 2, Fort Dodge). Our data revealed that functional T cells were needed to control the IBDV-antigen load in the acute phase of infection at 5 days post infection. The target organ of IBDV, the bursa of Fabricius, of Tx-CsA-birds had a significantly higher antigen load than the one of T cell-intact birds ($P < 0.05$). Tx-CsA-treatment abrogated the IBDV-induced inflammatory response and significantly ($P < 0.05$) reduced the incidence of apoptotic bursa cells and the expression of cytokines such as interleukin 2 (IL-2) and interferon-γ (IFN-γ) in comparison to T cell-intact birds. T cell-released IL-2 and IFN-γ may have mediated the induction of inflammation and cell death in T cell-intact birds. The IBDV-induced upregulation of tumor necrosis like-factor (TNF) expression was comparable between T cell-intact and Tx-CsA-birds. Tx-CsA-birds showed a significantly faster resolution of IBDV-induced bursa lesions than T cell-intact birds ($P < 0.05$). This study suggests that T cells modulate IBDV pathogenesis in two ways: a) they limit viral replication in the bursa in the early phase of the disease at 5 days post infection, and b) intrabursal T cells promote bursal tissue damage and delay tissue recovery possibly through the release of cytokines and cytotoxic effects.

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Introduction

Infectious bursal disease (IBDV), a member of the *Birnaviridae* family, whose genome consists of two segments of double-stranded RNA, causes an acute, highly contagious and immunosuppressive disease in young chickens [29]. The main target cells for IBDV replication are the actively dividing B lymphocytes; thus infection leads to the destruction of lymphoid cells in the bursa of Fabricius. Other lymphoid organs such as cecal tonsils are also affected by IBDV, but there virus replication and damage of lymphoid cells was less extensive and recovery was more rapid than in the bursa [40, 46]. The acute phase lasts one to two weeks and is often accompanied by variable mortality. In birds that survive the acute infection, virus replication subsides, and the depleted bursal follicles become repopulated with B cells and immune competence returns to levels of uninfected hatch mates [20]. The mechanisms of bursa destruction and subsequent recovery are not understood. Chickens infected with IBDV mount a vigorous anti-IBDV antibody response [29] that is critical for host defense.

Normal chickens have very few resident bursal T cells [26, 36, 44]. However, studies in our laboratory demonstrated that replication of IBDV in the bursa is accompanied by an influx of T cells at the site of virus replication [22, 44]. These IBDV-induced intrabursal T cells consist of both CD4+ and CD8+ T cells, which are activated and can proliferate *ex vivo* after stimulation with IBDV [22]. The role of these T cells in IBDV pathogenesis and recovery is not known. T cells may contribute to the induction of lesions. They were shown to induce severe tissue damage in Herpes simplex virus (HSV), HIV, or Coxsackievirus infections [5, 24, 25, 31]. Our objective was to study the role of T cells in IBDV-induced pathogenesis and recovery using a T cell-compromised chicken model. Our study suggests that T cells modulate IBDV pathogenesis by limiting viral replication in the bursa in the early phase at 5 days following infection. But intrabursal T cells also promoted bursal tissue damage and delayed tissue recovery.

Materials and methods

Chickens

Specific pathogen-free (SPF) chickens or embryonated eggs from HyVac (Gowrie, IA, USA) were used. Chickens were hatched and reared in Horsfall-Bauer-type isolation units for the duration of the study. The birds were given food and water *ad libitum*. Birds for different experimental groups were housed in separate isolation units.

Thymectomy and Cyclosporin A (Tx-CsA) treatment

At one day post hatch, chickens were surgically thymectomized following procedures previously published [7, 38]. Briefly, the chickens were anesthetized by intramuscular injection of 0.1 to 0.15 ml of sodium pentobarbital solution (16 mg/ml). After opening the neck via dorsal incision, thymus lobes running beside the carotid arteries were removed with forceps. The wound was closed with surgery clamps. Starting from 7 days after the operation, the birds were treated with 100 mg/kg body weight (BW) of Cyclosporin A (CsA) intramusculary every 3 to 4 days throughout the duration of the experiments [32]. One or two days following the 3rd CsA injection, chickens were examined for circulating T cells and mitogenic

responses. During the first 2 weeks of the experiment, chickens were given antibiotics in the drinking water to prevent bacterial infections.

Virus

A commercial vaccine strain of IBDV (IBDV-Bursine 2, Fort Dodge, IA, USA) of intermediate pathogenicity, which was propagated and titrated in embryonated chicken eggs, was used [40, 44, 45]. The intermediate IBDV strain induces significant infiltration of T cells in the bursa of Fabricius [44]. At 20 days post hatch, T cell-intact and Tx-CsA-birds were intraocularly inoculated with 10^3 embryo infectious dose $(EID)_{50}$/bird of IBDV.

H&E staining and in situ apoptosis assay

For the detection of histopathological lesions, the bursa of Fabricius was collected, fixed in 10% phosphate-buffered formalin and stained with hematoxilin and eosin (H&E). Lesions were observed microscopically and lesion scores were determined and compared between groups [20, 40].

The TUNEL method was applied for *in situ* detection of apoptotic cells in the bursa of Fabricius by using the Apoptac® Kit (Oncor) [45].

In situ detection of IFN-γ and IL-2 like factor

Oligonucleotides (of 20 nucleotides) were designed as antisense probes based on published cDNA sequences of IL-2-like factor [6, 43] and IFN-γ [9] (Table 1). The probes were used as an oligonucleotide cocktail of 3 twentymers per cytokine selected from conserved regions of the cytokine (Table 1). The probes were labeled with digoxigenin (DIG) following the protocol for DIG oligonucleotide tailing provided by the manufacturer (Roche). The *in situ* hybridization protocol followed previously published procedures by Njenga et al. [34]. Briefly, frozen tissues were sectioned and fixed in ice cold 4% paraformaldehye for 5 min. The slides were digested with 1 μg of proteinase K per ml at 37 °C for 30 min, were washed with phosphate buffer, were acetylated for 10 min in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride and were washed, dehydrated in ascending series of ethanol, and then air dried. Sections were incubated for 2 h at 37 °C in prehybridization solution as described [34]. After prehybridization, slides were incubated in a moist chamber over night at 37 °C with 100 μl/slide of hybridization solution containing a 1:150 dilution of the three DIG-labeled oligonucleotides (100 pmol of labeled oligonucleotide) for each cytokine detection. The hybridization solution consisted of 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 μg of tRNA per ml, 500 μg of fragmented salmon sperm DNA per ml, 1×Denhardt's solution, 600 mM NaCl, 1 mM EDTA and 10 mg/ml of yeast tRNA, 10% Dextran sulfate 0.01 M dithiothreitol (DTT) and 0.25% sodium dodecyl sulfate (SDS) [31]. After hybridization, the slides were washed twice for 15 min each with 2×SSC (0.3 M NaCl plus 0.3 mM sodium citrate) at 37 °C, twice with 1×SSC and twice with 0.25×SSC. Hybridized probes were detected with anti-DIG antibodies coupled to alkaline phosphatase and were developed according to the manufacturer's instruction (DIG Nucleic Acid Detection Kit, Roche). Cocktails of 3 DIG-labeled sense probes each were used as negative controls for hybridization (Table 1). These sense probes were designed based on the sense sequence of the IFN and IL-2 cDNA and corresponded to the oligonucleotides used as probes in this study (Table 1). The number of signal-positive cells was counted in 20 randomly chosen fields of two sections per bird at a magnification of 250×. A bird was considered to be positive for cytokine up-regulation when more than 2 positive cells were found per section.

Table 1. Oligonucleotide probes and primers used in this study

Primer/probe	Size (bp)	Direction	GenBank acc. no.	Sequence
IFN-γ1	20	antisense	U27465	5′-GAA TGA CTT GAG TTA AAG TC-3′
IFN-γ2	20	antisense	U27465	5′-TTT CTC TCT GTC CAG TTC TT-3′
IFN-γ3	20	antisense	U27465	5′-GAA GAG TTC ATT CGC GGC TT-3′
IL-2-1	20	antisense	AF017645	5′-ATA CAG CCA AAG ATC AGT AC-3′
IL-2-2	20	antisense	AF017645	5′-GTT GGT GTG TAG AGC TCG AG-3′
IL-2-3	20	antisense	AF017645	5′-CTC ACA AAG TTG GTC AGT TC-3′
IFN-γ4	20	sense	U27465	5′-GAC TTT AAC TCA AGT CAT TC-3′
IFN-γ5	20	sense	U27465	5′-AAG AAC TGG ACA GAG AGA AA-3′
IFN-γ6	20	sense	U27465	5′-AA GCC GCG AAT GAA CTC TTC-3′
IL-2-4	20	sense	AF017645	5′-GTA CTG ATC TTT GGC TGT AT-3′
IL-2-5	20	sense	AF017645	5′-TC GAG CTC TAC ACA CCA AC-3′
IL-2-6	20	sense	AF017645	5′-GAA CTC ACC AAC TTT GTG AG-3′
IBDV-1	26	sense	D10065	5′-ATA TAT GAA TTC GAT CGC ATC GAT GA-3′
IBDV-2	26	antisense	D10065	5′-CTC GAG TTA CCT TAT GGC CCG GAT TA-3′
GADPH-1	17	sense	M11213	5′-GGG TGG AAA GTC GGA GT-3′
GADPH-2	20	antisense	M11213	5′-GAA GAT AGT GAT GGC GTG CC-3′
IBDV-probe	26	sense	D10065	5′(FAM)-TCC CCT GAA GAT TGC AGG AGC ATT TG-(TAMRA)-3′
GADPH-probe	20	sense	M11213	5′(FAM)-CAA GTT TCC CGT TCT CAG CC-(TAMRA)-3′
TNF-1	21	sense	M80573	5′ACG CAC TCT CCA GCA AAC ATC-3′
TNF-2	20	antisense	M80573	5′-AAT CTG GTT GGG GTT CGG AG-3′

Activity assay for nitric oxide (NO)

Spleens from IBDV-infected and virus-free birds were harvested and crushed to make single cell suspensions of splenocytes [16, 17]. The splenocyte suspension was layered on Ficoll-Hypaque (Sigma, gradient density 1.090) and mononuclear cells were harvested after centrifugation at $3000 \times g$ for 10 min. 100 μl of 5×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and antibiotics were seeded in 96-well plates. The cells were treated with medium alone or with 10 μg/ml lipopolysaccharide (Sigma). After incubation for 48 h at 41 °C, the cell culture supernatants were harvested and tested. Nitrite concentrations were determined by mixing 100 μl cell culture supernatant with an equal volume of Griess reagent [15–17]. Sodium nitrite was used as a standard to determine NO concentrations. The results are expressed in μM of NO.

Immunohistochemistry assay to detect intrabursal T cells and IBDV

The procedures have been described [20, 44]. Bursae of Fabricius were collected, snap frozen in Tissue Tek® optimum cutting temperature compound (Miles Inc., Elkhart, IN, USA), sectioned and processed for immunohistochemical staining. T cells were examined using monoclonal anti-chicken CD3 antibody (Southern Biotech). A polyclonal rabbit anti-IBDV serum obtained from Dr. K. Tsukamoto, National Institute of Animal Health, Japan was used

for IBDV detection [44]. The group means of the number of IBDV infected cells or CD3+ cells per field at 400x were determined after counting 10 field/tissue/bird and compared.

Proliferation assay

Mitogenic responses of peripheral blood leukocytes were carried out as described previously [16, 27]. Briefly, 1:10 dilutions of heparinized whole blood samples were stimulated *ex vivo* with 100 μg of concanavalin A (ConA)/ml at 41 °C. After 43 h of incubation, the cells were pulsed with 1 μCi/well [^{3}H]-thymidine for 5 h, harvested and counted in the Matrix 9600^{TM} Direct Beta Counter (Packard Instrument Company, Meriden, CT).

Real time PCR

To quantitate the IBDV genome in the Tx-CsA and T cell-intact chickens, bursae of Fabricius were collected for real-time PCR (Perkin-Elmer) [32]. IBDV RNA from the tissues was isolated using a TRIzol^{TM} reagent (Life Technologies, Gaithersburg, MD). The total RNA was quantitated spectrophotometrically, reverse transcribed into cDNA using random hexamers (Superscript^{TM} First Strand Synthesis System for RT-PCR, Life Technologies), amplified and quantitated by real-time PCR using reagents from the TaqMan® PCR reagent kit (PE Applied Biosystems). The PCR mixture (50 μl) consisted of the following: 1× TaqMan® buffer, 3 mM $MgCl_2$ solution, 300 μM dATP, dCTP, dGTP, 600 μM dUTP, 200 nM of each IBDV-specific primer, or 200 nM of each GADPH-specific primer, 100 nM IBDV- or GADPH probe, 2.5 AmpliTaq Gold polymerase, 0.01 U/μl AmpErase UNG and 2 μl of the RT-product at a 1:10 and 1:100 dilution. GADPH was used as an internal standard. Primers used for amplification of the VP2 gene of IBDV and of GADPH as well as the probes are listed in Table 1. Each sample dilution was tested in triplicates for each primer set. The quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The GADPH and IBDV concentration per sample was calculated based on the threshold cycle value. The IBDV-genome load was determined by identifying the ratio of internal standard GADPH and IBDV.

Detection of tumor necrosis factor (TNF) upregulation by RT-PCR

TNF gene expression was determined by RT-PCR following standard procedures. Briefly, 5 μg of total bursal RNA was used for reverse transcription with Superscript^{TM} reverse transcriptase (Life Technologies) using random hexamers. Specific primers for chicken TNF sequence and internal standard control GADPH were used to detect TNF cDNA in the samples by PCR (Table 1). The PCR was conducted as follows: initial denaturation at 94 °C for 4 min, and 30 cycles of 94 °C for 1 min, annealing at 60 °C for 30 sec, polymerization at 72 °C for 1 min, and at the last cycle the reaction was extended at 72 °C for 10 min. The PCR products were electrophoresed, stained with ethidium bromide and analyzed.

Flow cytometric analysis of lymphoid cell populations

Single cell suspensions of bursa cells were separated in a discontinuous density gradient using Ficoll-hypaque [22]. We followed the protocol by Kim et al. [22] using directly FITC-labeled monoclonal anti-chicken CD3, anti-chicken CD4 and CD8 antibodies (Southern Biotechn.). Briefly, 10^6 cells were incubated with anti-chicken CD3-FITC, anti-chicken-CD4-FITC or anti-chicken CD8-FITC for 30 min on ice. Following three washes with phosphate buffered saline (PBS) containing 2% FBS, the cells were fixed with 4% paraformaldehyde and analyzed by the FacsCalibur (Becton Dickinson) and CellQuest software (Becton Dickinson). Viable lymphocytes were gated on the basis of forward and sideward scatter characteristics, and 10,000 events were analyzed for positive staining with FITC.

Table 2. Experimental procedure (Experiments 1 and 2)

Day	Events/Investigations
1 day PH	Tx
7–42 days PH	CsA-treatment
18–19 days PH	Flow cytometric analysis, mitogenic assay
20 days PH	IBDV-inoculation
1, 2, 3, 5, 8, 15, 22 days PI	Mitogenic assay, Pathology, Histopathology, immunohistochemical detection of IBDV, Cytokine detection
1, 2, 3, 5, 8, 15 days PI	Nitric oxide assay, CD3-immunohistochemical detection, IBDV-genome detection by Real time PCR, *in situ* apoptosis detection
15, 22 days PI	Antibody detection by ELISA

PH post hatch; *PI* post IBDV-infection

Serology

Serum samples were collected at 15 and 22 days post IBDV infection and anti-IBDV antibody titers were determined by using the IBDV antibody kit from Kirkegaard-Perry Laboratories (KPL) detecting anti-IBDV-antibodies from the IgG-type. Mean $\log_{10}$ IBDV antibody titers were calculated based on the manufacture's instructions and compared by Student's *t*-test.

Experimental procedure

In two repeat experiments (Table 2), 2.5 week old Tx-CsA-birds were tested for numerical and functional T cell suppression by flow cytometric analysis and mitogenic assay. T cell function was monitored by mitogenic assay through the duration of the experiment. During the peak of mitogenic inhibition in Tx-CsA-chickens, 25 birds of each group were infected intraocularly with 10^3 EID_{50} of IBDV or diluent. At days 1, 2, 3, 5, 8, and 15 (Experiments 1 and 2) and 22 (Experiment 1) post infection (PI), 5 birds per group were sampled and the following observations were made: a) antigen load in the Bursa of Fabricius by immunohistochemistry or real-time RT-PCR; b) pathological and histopathological lesions; c) cell death rate by *in situ* TUNEL assay; and d) up-regulation of cytokines such as TNF, IL-2 and IFN-γ by RT-PCR and *in situ* hybridization, respectively (Table 2).

Statistical analysis

Group responses within experiments were analyzed by Student's *t*-test, Kruskal Wallis test or chi-square test.

Results

Tx-CsA-treatment reduced the numbers and mitogenic responsiveness of circulating T cells but not the numbers and function of B cells

CsA-treatment has been known to selectively suppress T cell function by inhibiting expression of the IL-2 receptor and by blocking IL-2 mediated signal transduction [18, 48]. Tx-CsA induced a dramatic reduction ($P < 0.001$) in the mitogenic response of circulating chicken T cells, which lasted through the duration of the experiments (Fig. 1) up to 22 days post IBDV-infection (data not shown). Using the combination of surgical thymectomy and repeated CsA-treatment the number

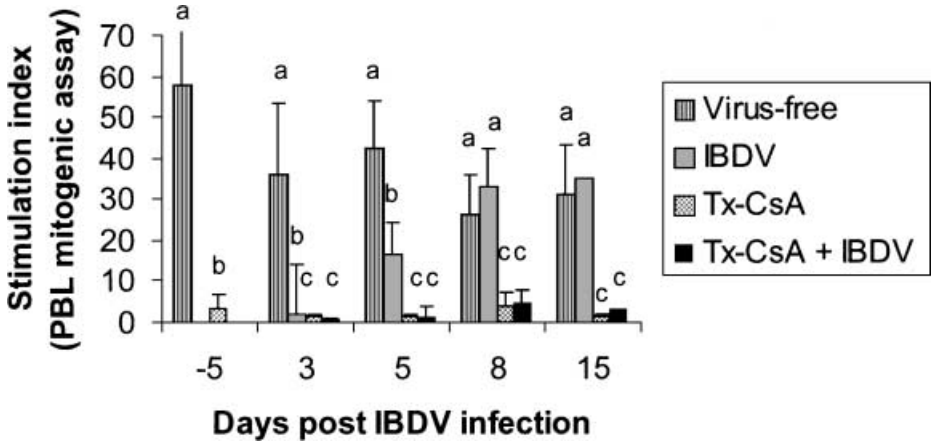


Fig. 1. Induction of T cell-immunodeficiency in chickens. Tx-CsA = thymectomized and CsA-treated chickens; PBL = peripheral blood leukocytes. At one day post hatch, chickens were surgically thymectomized and then treated intramuscularly every 3–4 days with 100 mg/kg body weight of Cyclosporin A (CsA). At various time points before and after IBDV infection, chickens were examined for mitogenic responses. A whole-blood mitogenic assay was performed using standard procedure. Presented is the average of stimulation indices (counts per minutes of ConA-stimulated cells/counts per minute of medium-incubated cells) of 10–20 chickens/group. Different superscript letters indicate significant differences ($P < 0.05$). The mitogenic response in the Tx-CsA-treated groups was still significantly suppressed at 22 days PI in comparison to T cell-intact controls (data not shown, $P < 0.05$)

of circulating CD4+ cells was reduced by 62±23 and CD8+ T cells by 62±20%. In contrast the number of IgM+ cells (B lymphocytes), as measured by flow cytometric analysis of pooled PBL samples, was not affected. Tx-CsA-treatment did not affect the ability of B cells to produce antibodies against IBDV at 15 days PI. At 15 days PI, IBDV-infected T cell-intact and Tx-CsA birds had serum anti-IBDV IgG levels of $\log_{10}$ of 3.8 ± 0.1 and 3.7 ± 0.1, respectively. Also at 22 days post infection there were no significant differences ($P > 0.05$) in anti-IBDV antibody production (data not shown). Virus-free birds did not have detectable anti-IBDV antibodies. Tx-CsA-treatment did not influence NO release by macrophages. In fact, spleen cells from IBDV-infected Tx-CsA-birds released significantly higher NO levels at 2 and 3 days PI after *ex vivo* stimulation with LPS than spleen cells from IBDV-infected T cell intact birds (Table 3; $P < 0.05$). Despite the reduction in circulating T cells, similar T cell accumulation was noted in the bursa of Fabricius of Tx-CsA-birds and T cell-intact chickens following IBDV infection (Fig. 2).

T cell cytokines such as IFN-γ and IL-2 are known to be up-regulated in the acute phase of IBDV infection [19, 22]. The *in situ* hybridization results presented in Table 4 confirmed this observation. Exposure of T cell-intact birds to IBDV resulted in up-regulation of IFN-γ and IL-2 expression in the bursa during the first three days of exposure. At one day PI, which was the day of peak expression,

Table 3. *Ex vivo* production of NO by spleen cells following LPS stimulation

Groups	NO (μM) (number of birds with significantly ($P < 0.05$) elevated NO production over controls/total number tested)			
	1 day PI	2 days PI	3 days PI	5 days PI
Virus-free	3.4 ± 1.4 (0/3)	2.1 ± 0.5^{a} (0/3)	2.8 ± 0.2^{a} (0/3)	2.7 ± 1.1 (0/3)
IBDV	1.6 ± 0.3 (0/5)	3.4 ± 1.4^{a} (1/5)	3.7 ± 1.6^{a} (3/5)	5.1 ± 5.1 (0/5)
Tx-CsA	2.0 ± 0.3 (0/3)	ND	4.1 ± 2.3^{a} (0/3)	2.7 ± 0.6 (0/3)
Tx-CsA-IBDV	2.5 ± 0.7 (0/5)	10.6 ± 10.4^{b} (4/5)	11.7 ± 10.1^{b} (5/5)	2.6 ± 0.4 (0/5)

T cell-intact and Tx-CsA-treated chickens were inoculated at 2.5 weeks of age with IBDV. At 1, 2, 3, and 5 days PI, single spleen cell suspensions were stimulated *ex vivo* with 10 μg/ml of LPS at 41 °C. After 48 h of stimulation the spleen cell supernatants were tested for NO with Greiss Reagent. At 8 and 15 days PI, none of the stimulated or unstimulated spleen cell cultures from infected birds released NO levels above control (data not shown). Different superscript letters indicate significant differences between groups ($P < 0.04$, Kruskal Wallis Test)

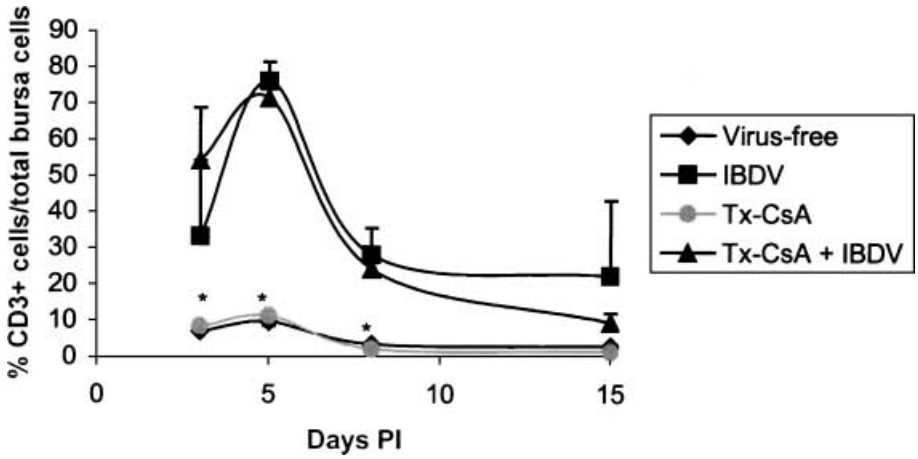


Fig. 2. Accumulation of T cell in the bursa of Fabricius at various time points post IBDV infection. At different day points following IBDV-infection, Tx-CsA-treated and T cell-intact birds were examined for T cells accumulating in the bursa of Fabricius by flow cytometric analysis. Presented is the average of the % of CD3+ cells in the bursa of Fabricius. Two repeat experiments were done with similar results. Presented is the result of one representative experiment. n = 5; $^{*}P < 0.05$

T cell-intact birds infected with IBDV had up to 24 IFN-γ and IL-2 signal positive cells per microscopic field at a magnification of 400x (data not shown). In addition, TNF-like factor mRNA was upregulated in T cell-intact birds as detected by RT-PCR (Table 4). Tx-CsA-treatment blocked detectable expression of IFN-γ or IL-2 mRNA in the bursa but TNF-like factor remained upregulated (Table 4) up to 22 days PI (data not shown). Birds unexposed to IBDV or those IBDV-infected for 8 to 22 days did not show any IL-2 and IFN-γ upregulation.

Table 4. Detection of IBDV-induced up-regulated intrabursal expression of IFN-γ, IL-2 and TNF-like factor

Groups	Number of birds positive for up-regulated cytokine mRNA at days PI[a]														
	1			2			3			5			15		
	IL-2	IFN-γ	TNF	IL-2	IFN-γ	TNF	IL-2	IFN-γ	TNF	IL-2	IFN-γ	TNF	IL-2	IFN-γ	TNF
Virus-free	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
IBDV	4/4	4/4	4/4	2/5	3/5	5/5	2/5	0/5	5/5	1/5	0/5	5/5	0/5	0/5	5/5
Tx-CsA	0/5	0/5	0/3	ND	ND	ND	0/3	0/3	0/3	0/3	0/3	0/5	0/3	0/3	0/5
Tx-CsA + IBDV	0/5	0/5	3/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5

T cell-intact and Tx-CsA-treated chickens were inoculated with IBDV. At 1, 2, 3, 5, and 15 days PI, bursa sections were used for *in situ* hybridization detection of IL-2-like factor and IFN-γ up-regulation. Birds were considered positive for IL-2 and IFN-γ upregulation when an average of more than two signal-positive cells were detected per section at 250×. From 8 days PI up to 22 days PI (data not shown), birds in all tested groups were negative for up-regulated IL-2-like factor and IFN-γ expression. Up-regulation of TNF-like factor mRNA expression was detected by RT-PCR using bursa samples collected at various time points post infection. Uninfected birds were negative for a TNF-like factor specific PCR product

[a]Expression of cytokine mRNA was detected by *in situ* hybridization or RT-PCR. *ND* Not done. At 8 and 22 days PI, all IBDV-infected birds were still positive for TNF-like factor up-regulation (data not shown)

Tx-CsA treatment resulted in increased IBDV genome and viral antigen load in the bursa of Fabricius

Immunohistochemical evaluation of bursa sections for IBDV-positive cells indicated that at 5 days PI the Tx-CsA-birds had a significantly higher IBDV-antigen load than T cell-intact birds ($P < 0.05$; Table 5). Before and after 5 days PI, there were no significant differences in IBDV-antigen load between groups (data not shown). This was confirmed by virus quantification using real time RT-PCR to detect the VP2 region of IBDV (Fig. 3). At 5 days PI, Tx-CsA-birds had a

Table 5. Immunohistochemical detection of IBDV antigen in the bursa of Fabricius

Treatment	IBDV	Number of birds/group	Average number of IBDV-positive cells/field (400 ×)	
			5 days PI	22 days PI[a]
None	−	3	0 ± 0^{a}	0 ± 0
None	+	5	56 ± 19^{b}	4 ± 4
Tx-CsA	+	5	106 ± 44^{c}	0.9 ± 2

Presented is the result of one of two repeat experiments that were conducted with similar results. Groups of immunologically intact and Tx-CsA-chickens were exposed to IBDV intraocularly. At 5 and 22 days PI, five birds per group were sacrificed and bursa sections were evaluated for IBDV antigen using immunohistochemistry [44]. Shown is the group average of IBDV-positive cells/microscopic field at 400 ×. Per bird we examined 10 randomly chosen microscopic fields. Different superscript letters indicate significant differences between groups ($P < 0.05$)

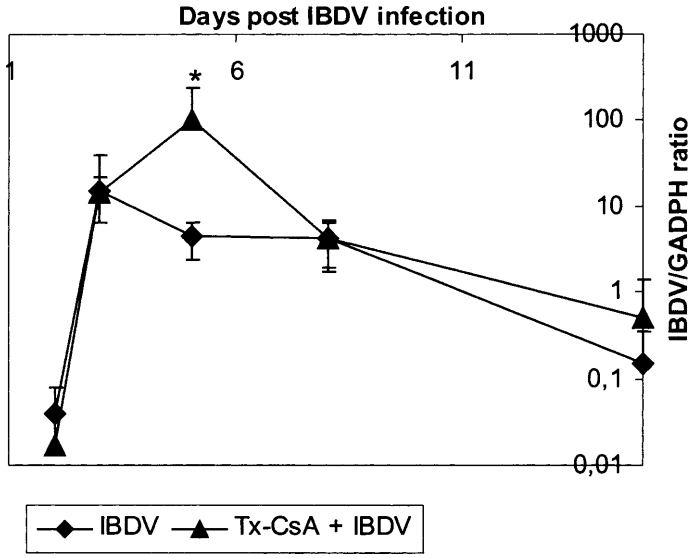


Fig. 3. Quantitation of IBDV genome using semi-quantitative real time RT-PCR. Groups of immunologically intact and Tx-CsA-chickens were exposed to IBDV intraocularly. At various time points during the infection, bursae of three to five birds per group were analyzed by real time RT-PCR for IBDV-viral load. Shown is the group average of the IBDV/GADPH expression ratio. * indicates significant differences between groups ($P < 0.05$)

significant higher viral RNA load than T cell-intact birds (Fig. 3). By 22 days PI, both Tx-CsA- as well as T cell-intact groups had cleared the virus from the bursa (Table 5).

Tx-CsA-treatment reduced IBDV-induced lesions and improved follicular recovery

Between 3 and 5 days PI, 70–80% of the infected T cell-intact as well as Tx-CsA-birds had grossly detectable lesions characterized by gelatination and necrosis of the bursa of Fabricius. Beginning at 5 days PI and lasting through the observation period of 22 days, T cell-intact as well as Tx-CsA-birds showed significantly lower bursa to body weight ratios in comparison to controls (data not shown). At 8 days PI, 2 of 9 birds in the T cell-intact group and 0 of 9 of the Tx-CsA-birds showed minor bursa gelatination (Table 6). At 15 days PI, none of the infected birds had detectable gross bursal lesions (Table 6).

Histopathological lesions in the bursa were characterized by inflammation and accumulation of heterophils, edema, depletion of bursa follicles, and cyste formation. Minor histopathological lesions were seen at 1 and 2 days PI in the T cell-intact as well as Tx-CsA-groups. At 5 days PI, follicular destruction was most pronounced. More than 90% of the bursa follicles were destroyed in both Tx-CsA and T cell-intact groups (Table 6). IBDV-infected T cell-intact birds developed a profound inflammatory response in the bursa of Fabricius as indicated by massive infiltration of heterophiles at 3 to 5 days PI, while Tx-CsA-birds had only a mild inflammatory response with few infiltrating heterophiles (Table 6).

Table 6. Bursa lesions following IBDV infection of T cell-intact and Tx-CsA-chickens

Days post IBDV infection	Number of birds with gross lesion/number bursa examined		Group mean of % of destroyed bursa follicles		Heterophile infiltration into the bursa	
	T cell-intact	Tx-CsA	T cell-intact	Tx-CsA	T cell-intact	Tx-CsA
2	0/4	0/5	0 ± 0	10 ± 22	$-^{*}$	$-$
3	7/10	8/10	$60 + 53$	76 ± 42	$+++$	$-$
5	7/10	7/10	100 ± 0	92 ± 18	$+++$	$+$
8	2/9	0/9	99 ± 3^{a}	87 ± 9^{b}	$-$	$-$
15	0/10	0/9	89 ± 15^{a}	65 ± 21^{b}	$-$	$-$
22	0/5	0/5	74 ± 12^{a}	40 ± 22^{b}	$-$	$-$

Summary of two repeat experiments. Groups of T cell-intact and Tx-CsA-treated birds were inoculated with IBDV. At 2, 3, 5, 8, 15 and 22 days PI, 3 to 5 birds from each group were sacrificed and the macroscopical and microscopical bursa lesions determined. Different superscript letters within each column indicate significant differences between groups ($P < 0.05$). $^{*}-$, no infiltration of heterophiles; $+$ infiltration of less than 50 heterophiles/field at $400\times$; $+++$, infiltration of more than 100 heterophiles/field at $400\times$. Different superscript letters within a row indicate significant differences ($P < 0.05$, Student's *t*-test). Uninfected T cell-intact and Tx-CsA birds were free from pathological and histopathological lesions at every time point tested during the experiment

Table 7. Restoration of bursa follicles over time after IBDV infection

IBDV-infected groups	% follicles with repopulation at days PI (number of birds with restoration of bursa follicles/number of birds per group)			
	5	8	15	22
T cell-intact	0 ± 0[a] (0/10)	1.1 ± 2.3 (2/10)	14 ± 16 (8/10)	26 ± 12 (5/5)
Tx-CsA	0 ± 0 (0/10)	12 ± 13* (5/8)	32 ± 21* (9/9)	60 ± 22* (5/5)

Intact and Tx-CsA-treated chickens were inoculated at 2.5 weeks of age with IBDV. At various time points after infection, bursa section stained with H&E were examined for follicle recovery expressed in % follicles that were more than 50% repopulated. [a]% follicles repopulated $\pm$ standard deviation. A value of 0 indicates that all the bursa follicles were more than 50% depleted of intrabursal cells and showed signs of inflammation. *$P < 0.03$. Presented is the summary of two experiments

Bursae from IBDV-infected chickens are known to recover from the infection and bursa follicles undergo lymphoid cell repopulation [20]. At 5 days PI, bursae of IBDV-infected T cell-intact as well as Tx-CsA chickens did not show recovery of destroyed bursa follicles (Table 7). At 8, 15 and 22 day PI, Tx-CsA-treated birds had higher recovery than intact birds ($P < 0.05$; Table 7, Fig. 4).

One aspect contributing to the delay in repopulation of bursa follicles in IBDV-infected T-cell-intact birds versus the Tx-CsA-birds may be an enhanced apoptotic cell death rate mediated by functional T cells. In this study, we determined the effect of Tx-CsA on the cell death rate using the *in situ* TUNEL assay [45]. The evaluation of the TUNEL assay indicated that T cell-intact birds had a significantly higher apoptosis rate at 8 days PI than Tx-CsA-birds ($P < 0.05$) (Figs. 5 and 6). At 15 days PI, T cell-intact and Tx-CsA-birds had comparable cell death rates.

Discussion

Previous studies have shown that IBDV-induced intrabursal T cells were activated and proliferated in *vitro* in response to stimulation with IBDV [22]. These T cells may have different roles in the pathogenesis of the disease. They may be important for clearance of the virus. They also may lead to immunopathological reactions mediated by cell-associated or cytokine-induced cytotoxicity [3, 5, 12, 14, 24, 30, 38, 41].

This study was conducted to understand the role of IBDV-induced intrabursal T cells in immunopathogenesis and tissue recovery using a T cell-compromised chicken model. SPF chickens were immunocompromised by surgical thymectomy and CsA treatment. This treatment reduced the numbers of circulating T cells and the mitogenic response of PBL. Tx-CsA-treatment did not affect the function of

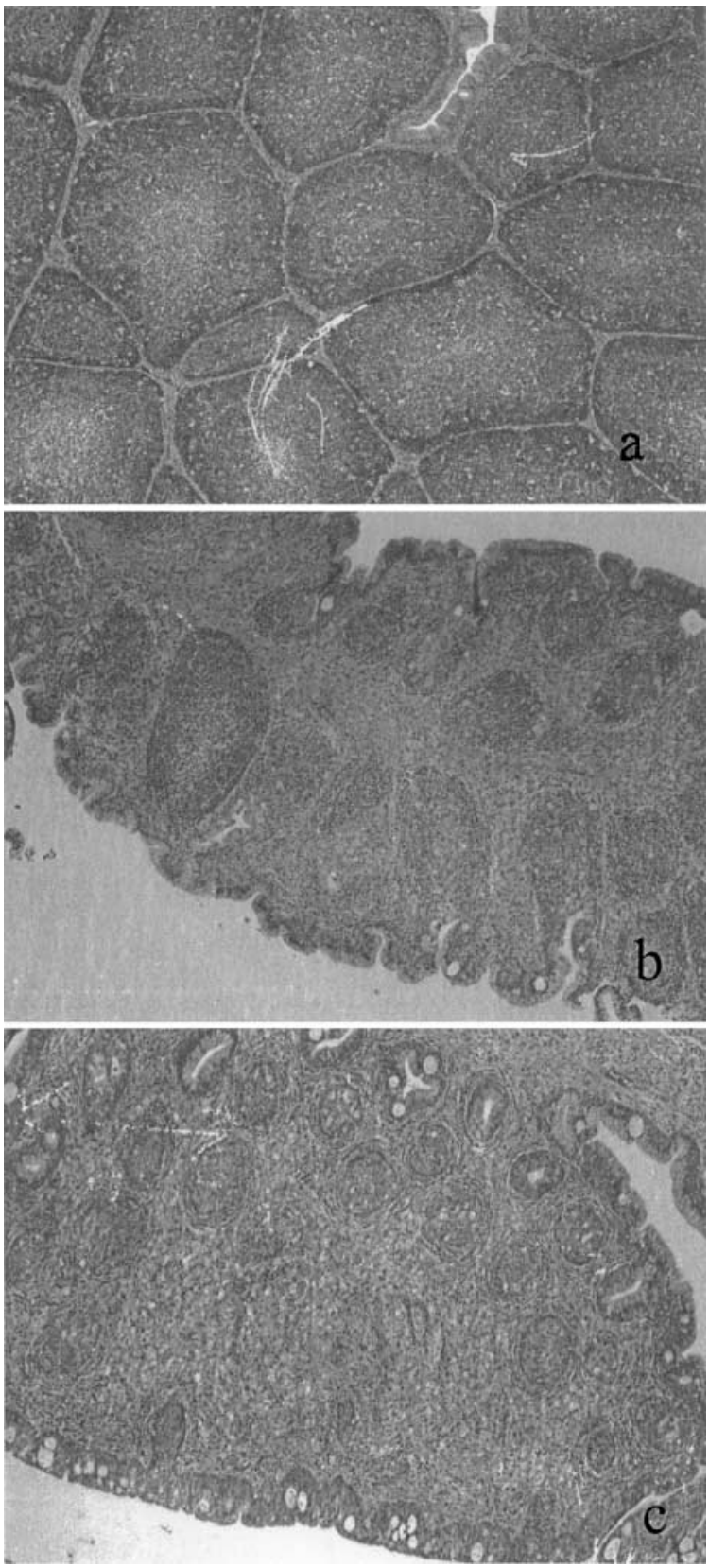


Fig. 4 (*continued*)

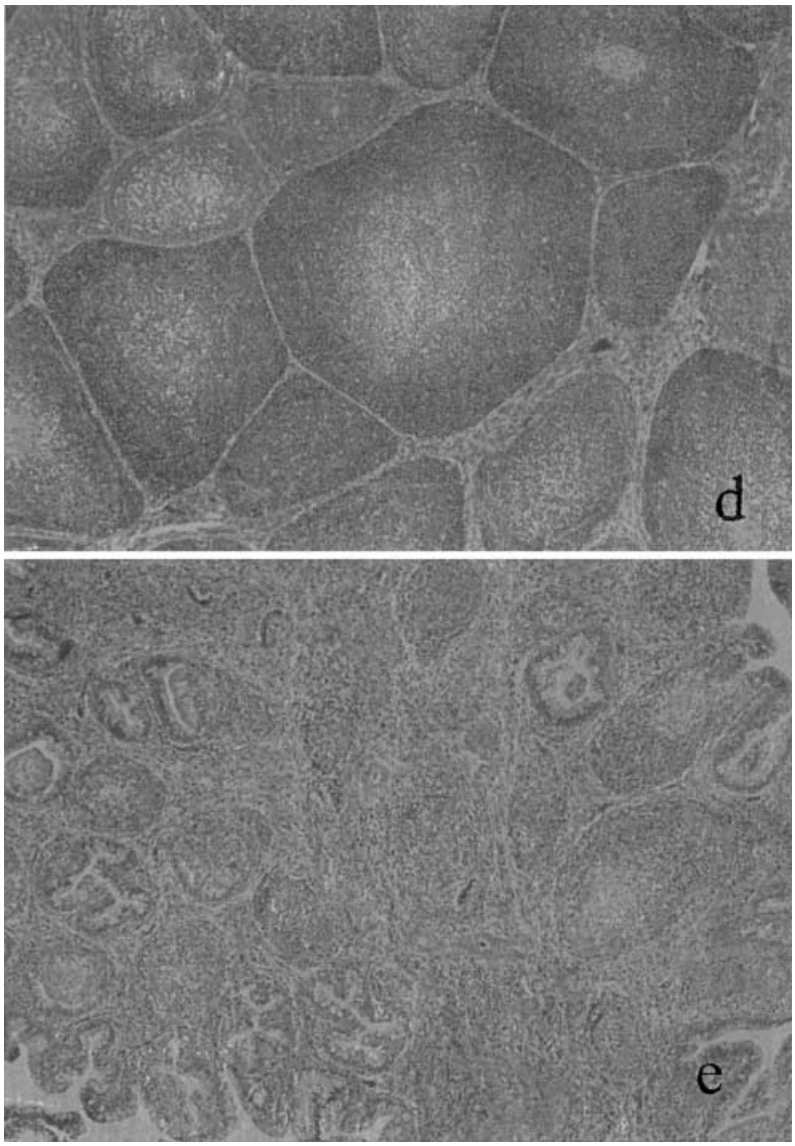
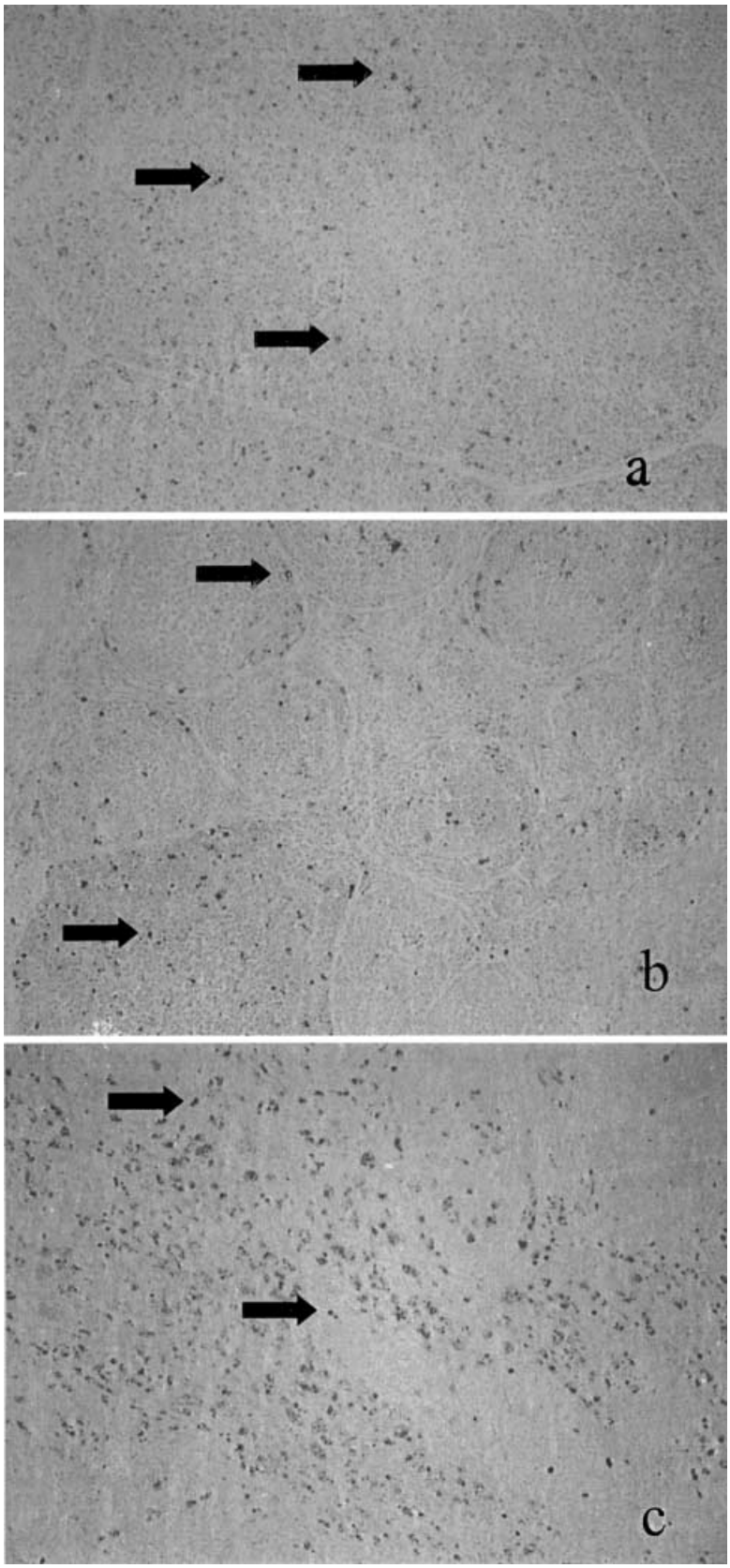


Fig. 4. Pathohistological bursa lesions (H&E staining) following IBDV-infection. **a** Bursa of an uninfected bird at 8 days PI (100x); **b** Tx-CsA-treated bird infected with IBDV at 8 days PI (100×); **c** IBDV-infected bird at 8 days PI (100x); **d** Tx-CsA-treated bird infected with IBDV at 15 days PI (100x); **e** IBDV-infected bird at 15 days PI (100×)

Fig. 5. *In situ* apoptosis staining of bursa sections from IBDV-infected and virus-free birds. **a** Virus-negative bird, dark dots indicate positively stained cells that undergo apoptosis or late stages of necrosis (arrows; 250×); **b** apoptosis staining of a bursa section from a Tx-CsA-treated IBDV-infected bird at 8 days PI (arrows indicate positive cells; 250×); **c** apoptosis staining of an IBDV-infected T cell-intact bird at 8 days PI (arrows indicate positive cells; 250×)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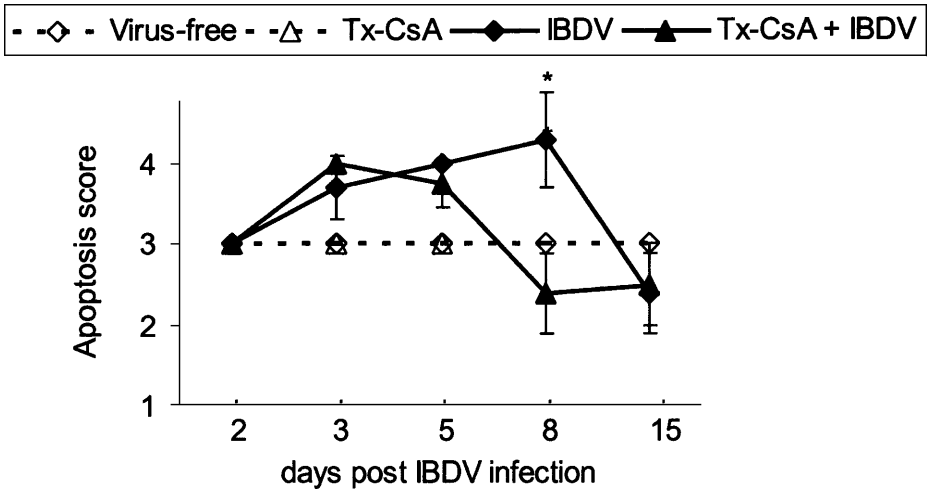

Fig. 6. Effect of IBDV-infection on the cell death rate detected by the *in situ* TUNEL assay in the bursa of Fabricius. The cell death rate was scored from 1 to 4 using microscopic evaluation of the tissue section. A score of 3 describes the cell death rate in the bursa of Fabricius of an uninfected T cell-intact or T cell-depleted bird which indicates 30 to 40% apoptotic and necrotic cells per total cells; score 4 an increase in cell death of $\geq$ 30%; score 2 a decrease of the *in situ* signal of $\leq$ 30% from uninfected controls; and score 1 a decrease of $\leq$ 50%. $P < 0.04$ by Student's *t*-test

B cells and macrophages. As shown by Kim et al. [22], at 5 days PI, T cell-intact birds had a significantly lower IBDV-antigen load in the bursa than Tx-CsA-birds. This observation indicated that the presence of functional T cells was important in the control of virus replication up to 5 days PI. After 5 days PI, there were no significant differences in virus-clearance between Tx-CsA and T cell-intact birds. How T cells mediated viral clearance is not known. In the early phase around 5 days PI, T-cell helper function may be needed to optimize B cell and macrophage activities to control virus infection [28]. In the late phase after 5 days PI, non-T cell-mediated mechanisms such as antibody-dependent cellular cytotoxicity [1, 8] as well as macrophage functions such as phagocytosis of infected cells may have participated in IBDV-clearance [11]. In fact, Tx-CsA-treated birds had significantly higher NO release in spleen cell cultures after LPS stimulation at 2 and 3 day PI than T cell-intact or virus-free control birds ($P < 0.04$). Possibly enhanced NO production may have contributed to viral clearance in Tx-CsA-treated birds. At this point we can not exclude the participation of natural killer (NK) cells in viral clearance although previous data indicated that NK cell-mediated cytotoxic activity was not elevated in IBDV-infected birds [40].

Of interest was our finding that IBDV-infection of T cell-intact birds led to a greater inflammatory response in the bursa than infection of Tx-CsA-birds. The inflammation in T cell-intact birds may have been mediated though T cell-released cytokines such as IL-2 and IFN-γ that were not expressed in Tx-CsA-birds. T cell cytokines are known to stimulate macrophages, to release inflammatory mediators and chemokines, and to initiate the influx of granulocytes [2, 10].

Our data showed that the expression of intrabursal TNF-like factor was equally up-regulated in T cell-intact and Tx-CsA-birds from day 1 PI up to the end of the experiment. TNF may be released by macrophages and play a role in tissue destruction and/or tissue recovery [4, 23].

We used the *in situ* TUNEL assay [45] to study the cell death rates from day 2 to day 15 PI. In the *in situ* TUNEL assay the terminal transferase (TdT) labels DNA fragmentation during the early stages of apoptosis, and to a low percentages also necrotic cells [13]. The evaluation of the TUNEL assay indicated that Tx-CsA-birds had significantly lower apoptosis rates at 8 days PI than T cell-intact birds ($P < 0.05$). The enhanced bursa cell destruction mediated by IBDV-induced T cells at 8 days PI may be mediated by cytotoxic T cells. Previous studies demonstrated that cytotoxic CD4+ and/or CD8+ T cells destroy not only virus-infected but also uninfected bystander cells [12, 41]. Cytotoxic T cells accelerate the lysis of cells expressing viral antigens [33]. Non-specific mechanisms such as T cell-released cytokines or T cell-activated macrophages may also enhance the cell death rate in the bursae of infected birds. We noted previously that the IBDV-induced bursal T cells inhibited mitogenic proliferation of normal splenocytes [21]. This observation indicated suppressive or cell-destructive effects of intrabursal T cells on uninfected by-stander cells. Of interest was that the suppressive effect of T cells was mediated by cell-to-cell contact as well as by cell-free supernatant [21]. Further studies are needed to understand T cell-induced destruction of bursal lesions in IBD.

Between 8 and 22 days PI, Tx-CsA-birds showed a significantly higher number of repopulating bursa follicles than T cell-intact birds ($P < 0.05$). This observation indicated that the presence of functional T cells may have delayed recovery of IBDV-induced depletion of bursal follicles. One aspect contributing to the delay in repopulation of bursa follicles in IBDV-infected T cell intact birds may be an enhanced apoptosis rate mediated by functional T cells.

The above findings suggest that T cells modulate IBDV pathogenesis in two ways: a) they limit viral replication in the bursa in the early phase at 5 days PI [37], b) they promote bursal tissue damage and bursa recovery possible though the release of inflammatory cytokines and cell-mediated cytotoxic effects [24, 30]. No studies were done so far demonstrating the suppressive effects of T cells in recovery in infectious diseases. Some evidence was provided by studies of non-infectious diseases such as pouchitis. In pouchitis, which is a complication in the recovery phase following restorative proctocolectomy, it was demonstrated that activated mucosal T cells and IFN-γ production might lead to mucosal destruction and crypt hyperplasia [42]. In another study, Valdimarsson et al. [47] demonstrated that eruption of psoriatic skin lesions coincides with epidermal infiltration and activation of T cells while spontaneous or treatment-induced resolution of the lesions is preceded by the reduction or disappearance of epidermal T cells [47].

Further studies are needed to understand the role of T cells in the recovery from virus-induced lesions. Adoptive transfer experiments and T cell-depletion models, in which T cell populations will be depleted selectively, will give more insights

in what kind of T cell mechanisms influence virus-induced immunopathogenesis and recovery.

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