Immunological Characterization of Intraocular Lymphoid Follicles in a Spontaneous Recurrent Uveitis Model

Kristina J. H. Kleinwort,[1] Barbara Amann,[1] Stefanie M. Hauck,[2] Regina Feederle,[3] Walter Sekundo,[4] and Cornelia A. Deeg[1,5]

[1]Institute of Animal Physiology, Department of Veterinary Sciences, LMU Munich, München, Germany
[2]Research Unit for Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health GmbH, München, Germany
[3]German Research Center for Environmental Health GmbH, Core Facility Monoclonal Antibody Development, München, Germany
[4]Clinic for Ophthalmology, UKGM Marburg, Marburg, Germany
[5]Experimental Ophthalmology, Philipps University of Marburg, Marburg, Germany

Correspondence: Cornelia A. Deeg, Experimental Ophthalmology, Philipps University of Marburg, Baldingerstrasse, D-35033 Marburg, Germany;
Cornelia.Deeg@uni-marburg.de.

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Purpose. Recently, formation of tertiary lymphoid structures was demonstrated and further characterized in the R161H mouse model of spontaneous autoimmune uveitis. In the horse model of spontaneous recurrent uveitis, intraocular lymphoid follicle formation is highly characteristic, and found in all stages and scores of disease, but in depth analyses of immunologic features of these structures are lacking to date.

Methods. Paraffin-embedded eye sections of cases with equine spontaneous recurrent uveitis (ERU) were characterized with immunohistochemistry to gain insight into the distribution, localization, and signaling of immune cells in intraocular tertiary lymphoid tissues.

Results. Ectopic lymphoid tissues were located preferentially in the iris, ciliary body, and retina at the ora serrata of horses with naturally-occurring ERU. The majority of cells in the tertiary lymphoid follicles were T cells with a scattered distribution of B cells and PNA^+ cells interspersed. A fraction of T cells was additionally positive for memory cell marker CD45RO. Almost all cells coexpressed CD166, a molecule associated with activation and transmigration of T cells into inflamed tissues. Several transcription factors that govern immune cell responses were detectable in the tertiary lymphoid follicles, among them Zap70, TFIIB, GATA3, and IRF4. A high expression of the phosphorylated signal transducers and activators of transcription (STAT) proteins 1 and 5 were found at the margin of the structures.

Conclusions. Cellular composition and structural organization of these inflammation-associated tertiary lymphoid tissue structures and the expression of markers of matured T and B cells point to highly organized adaptive immune responses in these follicles in spontaneous recurrent uveitis.

Keywords: spontaneous recurrent uveitis, autoimmune, inflammation, tertiary lymphoid tissue, lymphoid follicle

Recurrent uveitis is an intraocular autoimmune disease characterized by inflammatory attacks of various duration and severity.[1] In Europe and the United States, 10% of blindness in working age adults is caused by uveitis.[2] All different clinical forms of uveitis in man (anterior, intermediate, posterior, or panuveitis) are characterized by an inflammatory cellular infiltrate in the inner eye.[3] Several animal models contributed to the understanding of uveitis pathophysiology. In rodents, different uveitis models can be induced experimentally by injection of retinal proteins (S-antigen or interphotoreceptor retinoid binding protein [IRBP])[4] in combination with complete Freund's adjuvant, that ensures an aggressive, inflammatory immune response. With these models, the significant importance of $CD4^+$ T cells for uveitis pathology was proven with adoptive transfer experiments.[5]

Lately, spontaneous disease models were established in mice through transgenic expression of retinal proteins or neoantigens, partly accompanied by T-cell receptor (TCR) transgenic T cells or by human HLA.[6,7] The clinical features and pathology of these animal models were highly similar between some human conditions and the mouse.[7] Mice transgenic for the IRBP-specific TCR (R161H) with the uveitis-susceptible B10.RIII background spontaneously develop ocular inflammatory disease similar to human autoimmune uveitis.[8] The R161H uveitis model shows a spontaneous early onset of clinical disease with high incidence and a chronic progressive character.[8] Moreover, it is a valuable source for retina-specific T cells for cellular studies.[8] The disease is characterized by inflammation of retina and choroid leading to loss of vision through retinal damage.[8] Additionally, approximately 40% of R161H mice have lymphoid aggregates in the retina.[9] These aggregates develop 1 month after onset of clinical disease and organize to large lymphoid structures.[9] These lymphoid structures fit the criteria of tertiary lymphoid structures, as shown by gene expression studies and immunohistochemical analyses of cellular markers and transcription factors.[9] Inter-

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estingly, we also have detected lymphoid follicle-like structures in a high percentage of horses with equine recurrent uveitis (ERU), a naturally developing uveitis model. Prevalence of ERU varies enormously depending on the continent. Whereas in Europe up to 10% of horses suffer from ERU, in the United States up to 25% are affected.[10] Equine recurrent uveitis can develop at any age, but most horses show the first initial uveitis episode between four and six years of age.[11] The disease is accompanied by spontaneously occurring, remitting, and painful inflammation of the inner eye[11] and shares many clinical and pathologic features to the disease in man.[12] Affected horses show blepharospasm, lacrimation, and photophobia as well as periocular swelling, corneal edema, or miosis.[11,13] Formation of a hypopyon due to breakdown of the blood–retinal barrier and resulting immigration of autoreactive T cells into the inner eye is a further symptom of ERU.[14,15] Therapy with anti-inflammatory drugs (steroids) and cell immunosuppressants (cyclosporine) reveals an improvement of clinical symptoms.[14] With increasing number, the inflammatory episodes lead to irreversible damage of intraocular structures.[16] At terminal stage, the inflammatory process leads to blindness of the affected eye due to destruction of retinal architecture or retinal detachment and phthisis bulbi.[13,12] The horse model offers the remarkable possibility to proof pathophysiologic concepts in experimental animals of the same species,[17,18] since pathophysiologic concepts examined in the spontaneous model can be verified experimentally. For example, IRBP-induced uveitis in experimental horses replicates spontaneous ERU in many aspects.[18] In previous studies, lymphoid aggregates in the inner eye were a common finding in horses with experimental, IRBP-induced uveitis.[18] The finding that genuine tertiary lymphoid tissues develop within the retina in R161H mice[9] prompted us to perform in depth immunologic characterization of respective structures detectable in ERU, another valuable spontaneous recurrent uveitis model.

Methods

Specimens

For this study, a total of 7 healthy control and 7 ERU eyes were used. Equine recurrent uveitis specimens were derived from horses that were treated in the equine clinic and diagnosed with ERU according to clinical criteria as described.[19] Regarding uveitis score, one ERU horse was classified as mildly, two as moderately, and four as severely uveitic. The horses were between 3 and 12 years of age. Control and ERU eyes used for this study were either from animals that were euthanized due to causes unrelated to this study (control and ERU eyes) or that were enucleated for therapeutical reasons (ERU eyes). The horses did not receive any medication in advance to euthanasia or enucleation. All animals were treated according to the ethical principles and guidelines for scientific experiments on animals according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. No experimental animals were involved in this study.

Embedding of Eyes

Intraocular sections were prepared in well-defined sectors as described.[20] In brief, after cutting of the cornea and enucleation, remaining eyecups were sectioned into four pieces. Thereby we were able to receive all parts of the retina, including the ora serrata and also the transition to the adjacent anterior structures, ciliary body, and iris. After slicing, the eyecups were fixed in Bouin's solution (Sigma-Aldrich Corp., Taufkirchen, Germany) and subsequently dehydrated in series of alcohols. Later, tissue blocks were embedded in paraffin, sectioned (8 μm), and mounted on coated slides (Superfrost Plus; Medite, Burgdorf, Germany).

Antibodies

For characterization of tertiary lymphoid tissues present in eyes of horses with spontaneous recurrent uveitis,[21] we detected immunologic cell markers and transcription factors in paraffin-embedded eye sections of respective cases and healthy controls with immunohistochemistry. Exact cellular expression and coexpression were detected with multilabeling immunohistochemistry. The following antibodies were used: anti-CD45RO (clone OPD4, mouse monoclonal, dilution 1:50; DakoCytomation, Hamburg, Germany), anti-CD3$_\varepsilon$ (clone CD3-12, self-made monoclonal rat anti-cytoplasmatic domain of CD3; neat),[22] anti-CD4 (clone 3C6, self-made monoclonal rat anti-intracytoplasmatic domain of CD4; neat), anti-equine B cells (clone 13G7, self-made monoclonal rat anti-horse Ig light chain; neat), anti-phospho (Tyr701)-STAT1 (rabbit polyclonal, 1:100; Sigma-Aldrich Corp.), anti-phospho (Tyr705)-STAT3 (rabbit polyclonal, 1:100; Cell Signaling, Darmstadt, Germany), anti-phospho (Tyr694)-STAT5 (rabbit polyclonal, 1:100; Cell Signaling), anti-phospho (Tyr641)-STAT6 (goat polyclonal, 1:200; Santa Cruz Biotechnology, Heidelberg, Germany), anti-IRF4 (rabbit polyclonal, 1:100; Santa Cruz Biotechnology), anti-GATA3 (goat polyclonal, 1:200; Santa Cruz Biotechnology), anti-ZAP70 (rabbit polyclonal, 1:100; Cell Signaling), peanut agglutinin-biotin (1:300; Biozol, Eching, Germany), anti-Ki67 (clone MIB-1, mouse monoclonal, 1:100; DakoCytomation), anti-general transcription-factor IIB (TFIIB; rabbit polyclonal, 1:100; Santa Cruz Biotechnology), and anti-ALCAM (CD166; rabbit polyclonal, 1:100; Santa Cruz Biotechnology). Secondary antibodies were taken accordingly. These were goat anti-rabbit IgG coupled to Alexa 568, goat anti-mouse IgG Alexa 568, goat anti-rat IgG Alexa 568, donkey anti-goat IgG Alexa 568, streptavidin Alexa 568 (dilution 1:500; all from Invitrogen, Karlsruhe, Germany), mouse anti-rat IgG1 Cy5, or IgG2a FITC (dilution 1:200). To visualize lymphoid-like follicle location sites, sections were stained with hematoxylin and eosin (H&E).

Immunohistochemical Characterization

Before immunohistochemical staining, tissue sections were deparaffinized with xylol. Then, sections were rehydrated in descending alcohol series. Heat antigen retrieval was performed in citrate buffer pH 6.0 followed by EDTA buffer, pH 8.0, at 99°C for 15 minutes each. To prevent unspecific antibody binding, sections were first blocked with 1% BSA in Tris buffered saline–tween (TBS-T; 150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% Tween20) containing 5% blocking serum for 40 minutes at room temperature. Blocking serum was chosen according to the species in which the secondary antibody was produced. For multiple labeling, blocking steps were applied before each primary antibody incubation. Sections were sequentially incubated with primary antibodies (overnight at 4°C), always followed by respective secondary antibodies (30 minutes at room temperature [RT]). Finally, the sections were mounted with glass coverslips using fluorescent mounting medium (Carl Roth, Karlsruhe, Germany). Fluorescent images were recorded with Axio Imager M1 or Z1 and further analyzed with software Axio Vision 4.6 (Zeiss, Göttingen, Germany). For all stainings, isotype controls of irrelevant specificity were performed. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen).

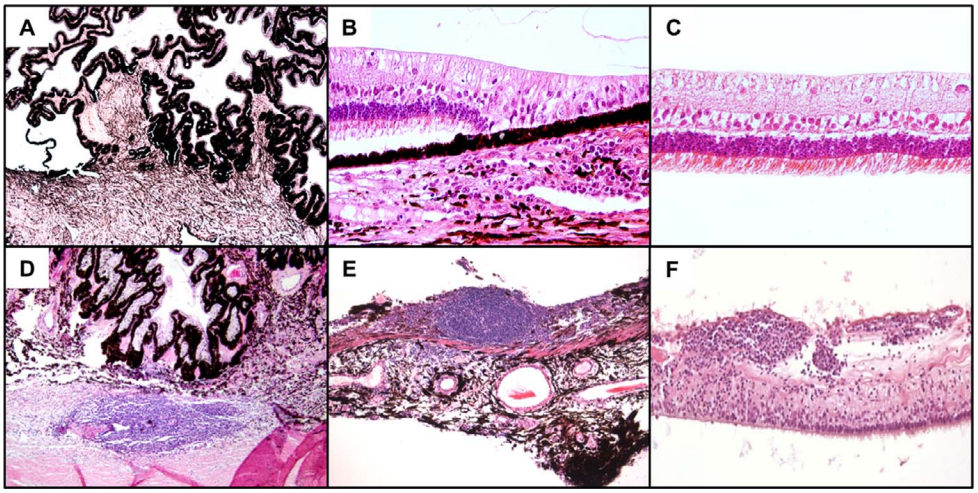

FIGURE 1. Equine recurrent uveitis eyes (**D–F**) show tertiary lymphoid-like follicles at typical localizations, whereas healthy horses (**A–C**) do not have lymphoid follicles in the inner eye. Hematoxylin and eosin staining (×20). (**A**, **D**) Iris stroma. (**B**, **E**) Ora serrata. (**C**, **F**) Retina.

RESULTS

In horses with naturally occurring spontaneous ERU, tertiary lymphoid tissues develop regularly at multiple sites within the eyes (Figs. 1D–F), in contrast to eyes of healthy horses (Figs. 1A–C), where no assimilable lymphoid tissues evolve. Major anatomic sites for these follicles in uveitic eyes are the iris stroma (Fig. 1D), the retina (Figs. 1F, 2C–D) with ora serrata (Fig. 1E), and the ciliary body (Figs. 3–5). The immune cell composition of the follicles was equal, independently of their localization. Further characterization of the immune cell composition with immunohistochemistry confirmed a T-cell rich microenvironment in respective lymphoid-like follicles[21] (Fig. 3A; $CD3^+$ cells, green) with a scattered pattern of B cells (Fig. 3B; B cells, red) in eyes of ERU cases. Interspersed in the follicles, large cells staining positive for germinal center marker peanut agglutinin (PNA; Fig. 3E; PNA, red) were found. The majority of the T cells were $CD4^+$ T-helper cells (Fig. 3G; CD4, green). Of these, a subpopulation proliferated (Fig. 3H; $Ki67^+$ cells, red) as well as some $CD4^-$ lymphocytes (Fig. 3I; merged image of $CD4^+$ and $Ki67^+$ stainings, overlay, yellow). Approximately half of the $CD4^+$ (Fig. 3J) and some of the $CD8^+$ cells (Fig. 3M) coexpressed CD45RO (Figs. 3K, 3N), a marker for memory cells (Figs. 3L, 3O; overlays result in yellow color).

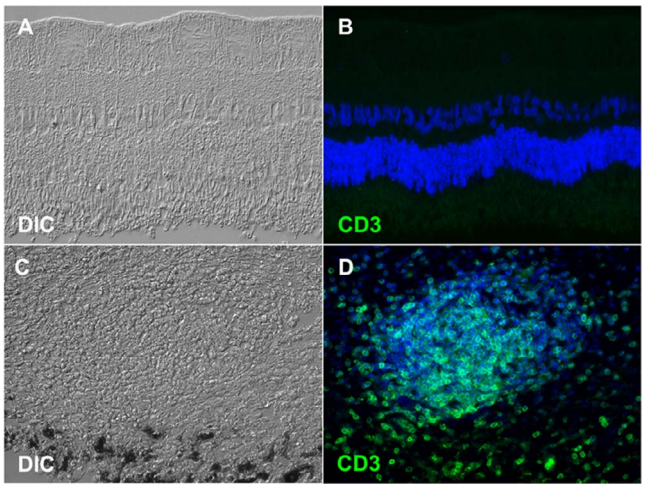

FIGURE 2. Immunohistochemical analyses confirm exclusive existence of tertiary lymphoid follicles in ERU eyes (×40). Differential interference contrast (DIC) image of a control (**A**) and ERU (**C**) retina. Staining with anti-$CD3_\varepsilon$ antibody (*green*) shows absence of an intraocular lymphoid-like follicle in control (**B**), but a clear follicle formation of T cells in ERU retina (**D**). Cell nuclei are stained with DAPI (*blue*).

Analyses of transcription factors associated with immune cell function in situ in these follicles revealed some $Zap70^+$ T cells (Fig. 4B; Zap70, red and Fig. 4C; Overlay $CD3^+$ and $Zap70^+$, yellow) at the margins of the lymphoid aggregates. Transcription factor IIB (TFIIB; Fig. 4E; TFIIB, red) was detectable in a scattered pattern throughout the lymphoid-like follicles, expressed by T cells (Fig. 4D; CD3, green and Fig. 4F; Overlay $CD3^+$ $TFIIB^+$, yellow). A similar pattern was observed for GATA3 (Fig. 4N; GATA3, red), which was expressed only in $CD4^+$ T cells (Fig. 4M; CD4, green and Fig. 4O; Overlay $CD4^+$ $GATA3^+$, yellow). Interestingly, signal transducers and activators of transcription (STAT) proteins 1 and 5 were found phosphorylated at the cortex zone of the follicles (Fig. 4H; pSTAT1, red and Fig. 4K; pSTAT5, red), expressed exclusively by T cells (Figs. 4G, 4J; $CD3^+$ cells, green; Fig. 4I; Overlay $CD3^+$ $pSTAT1^+$, yellow and Fig. 4L; Overlay $CD3^+$ $pSTAT5^+$, yellow).

Remarkably, the majority of the cells in the follicles were $CD166^+$ (Fig. 5B; CD166, red), irrespective if they were T or B cells (Fig. 5A; B cells, green). Further, $IRF4^+$ cells were evenly spread in the tertiary lymphoid tissues of spontaneous ERU cases (Figs. 5E, 5H, 5K; IRF4, red). IRF4 was expressed by an analog fraction of B cells (Fig. 5G; B cells, green and Fig. 5I; Overlay of B cells and IRF4, yellow) and of T cells (Fig. 5D; CD3, green and Fig. 5F; Overlay of $CD3^+$ T cells and IRF4, yellow). However, only few PNA^+ cells (Fig. 5J; PNA, green) also costained for IRF4 (Fig. 5K; IRF4, red and Fig. 5L; merged image of PNA and IRF4 stainings).

DISCUSSION

In a novel spontaneous model of recurrent uveitis, IRBP-TCR transgenic R161H mice develop genuine tertiary lymphoid tissue in the retinas.[9] Since we detected similar structures in eyes from horses with spontaneous and IRBP-induced uveitis, but only characterized a high percentage of T cells in these arrangements several years ago,[18,21] we were now interested to profoundly characterize these lymphoid follicle-like tissues in the natural uveitis model to gain further insights into the immune pathology of recurrent uveitis.

In both animal models, the R161H mouse and ERU-diseased horses, well-defined tertiary lymphoid tissues were detectable

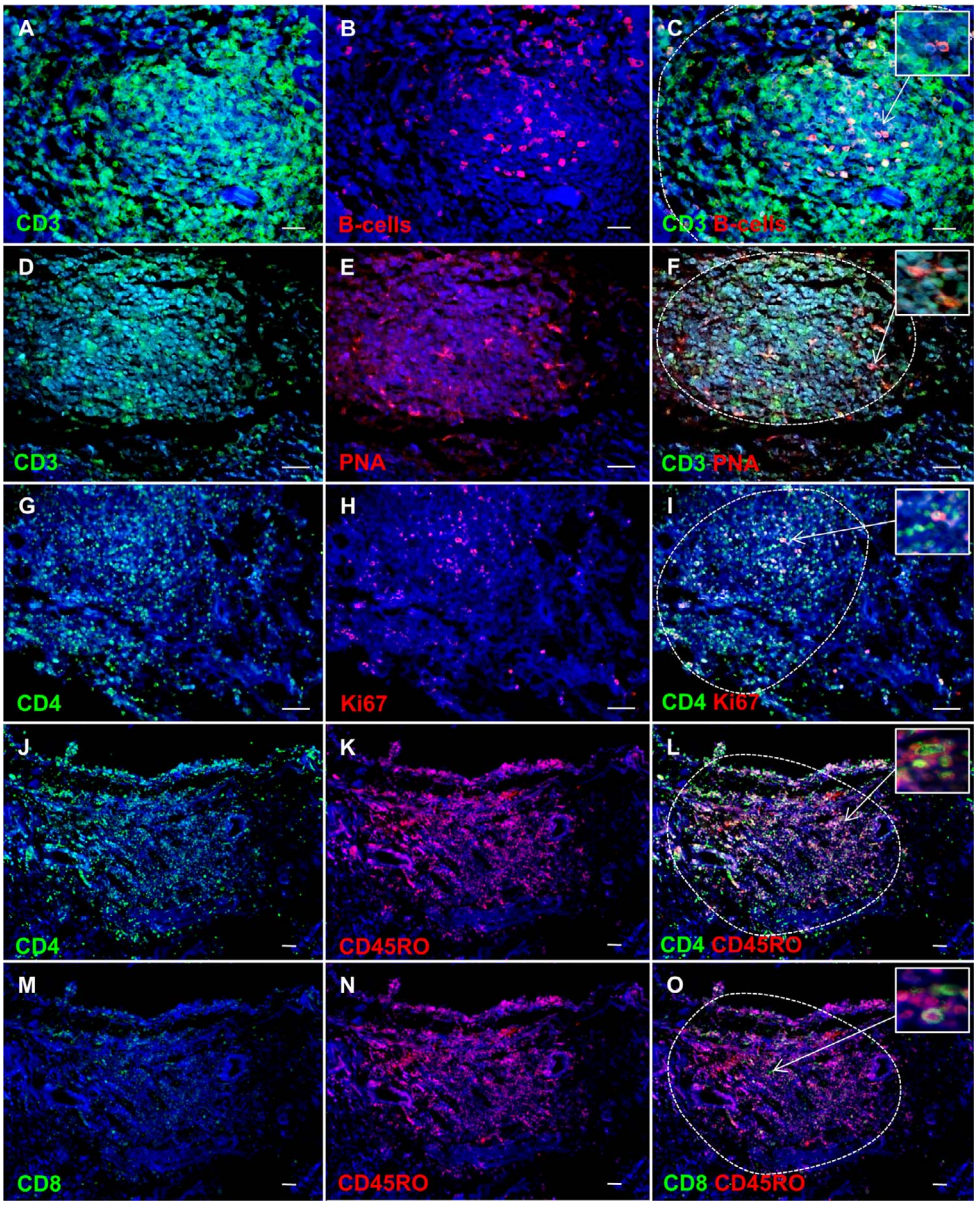

FIGURE 3. Immunohistochemical analyses of intraocular lymphoid aggregates reveal a T-cell rich composition with scattered B cells. (**A–O**) Characterization of cells, that form lymphoid-like follicles. (**A**) Majority of cells are T cells ($CD3^+$, *green*). (**B**) Interspersed B cells (Ig^+, *red*). (**D**) T cells (*green*) are surrounded by (**E**) cells staining positive for germinal center marker PNA (*red*). (**G**) Large population of T cells is $CD4^+$ (*green*). (**H**) A subpopulation of these $CD4^+$ cells and some $CD4^-$ cells proliferate (Ki67, *red*). (**J**) A larger subpopulation of $CD4^+$ cells (*green*) is positive for (**K**) memory cell marker CD45RO (*red*). (**M**) Some $CD8^+$ cells (*green*) also are double positive for (**N**) CD45RO (*red*). (**C**, **F**, **I**, **L**, **O**) Merged pictures of respective single stainings, overlay results in *yellow color*. Cell nuclei are stained with DAPI (*blue*). Location of lymphoid follicles is marked with a *white dashed line*. *White scale bars*: Length of 20 μm (magnification: ×20 [**J–O**] or ×40 [**A–I**]). Mapped follicles are located in ciliary body.

frequently in uveitic eyes, but the main localization of the follicles differed. Whereas R161H mice preferentially have intraretinal tertiary lymphoid tissues,[9] these structures are mainly built in the iris stroma (Fig. 1D), ora serrata (Fig. 1E), or at the ciliary body (Figs. 3–5) in natural-developing ERU. Nevertheless, the formed aggregates were structurally quite similar and resembled secondary lymphoid organs in the eyes of both uveitis models[9] (Fig. 1).

The further immunologic characterization revealed certain similarities in involved immune cells. The lymphoid aggregates in eyes of R161H mice and ERU cases contained analogous proportions and distributions of germinal center marker PNA^+ cells and of proliferation marker $Ki67^+$ cells[9] (Figs. 3F, 3I). GATA3, the master transcription factor for Th2 immune response,[23] was only detectable in a clear subfraction of $CD4^+$ T cells (Fig. 4M; $CD4^+$ cells, green; Fig. 4N; $GATA3^+$ cells, red; Fig. 4O; Overlay $CD4^+$ $GATA3^+$, yellow). Further, we detected Zap70, which is responsible for T-cell receptor signaling,[24] exclusively in T cells at the margin of the follicles in ERU eyes (Fig. 4C). This also agrees with the findings of pZAP70 in the follicles in eyes of R161H mice.[9] A large proportion of cells in the tertiary lymphoid tissues of R161H mice were positive for B220, a B-cell specific CD45 submarker. We tested for CD45RO, a marker of memory T and B cells, and detected a high expression rate in the lymphocytic aggregates in ERU eyes (Figs. 3K, 3N). Approximately half of the $CD4^+$ and some $CD8^+$ T cells were CD45RO double positive (Figs. 3L, 3O) with a remaining proportion of $CD45RO^+$ B cells.

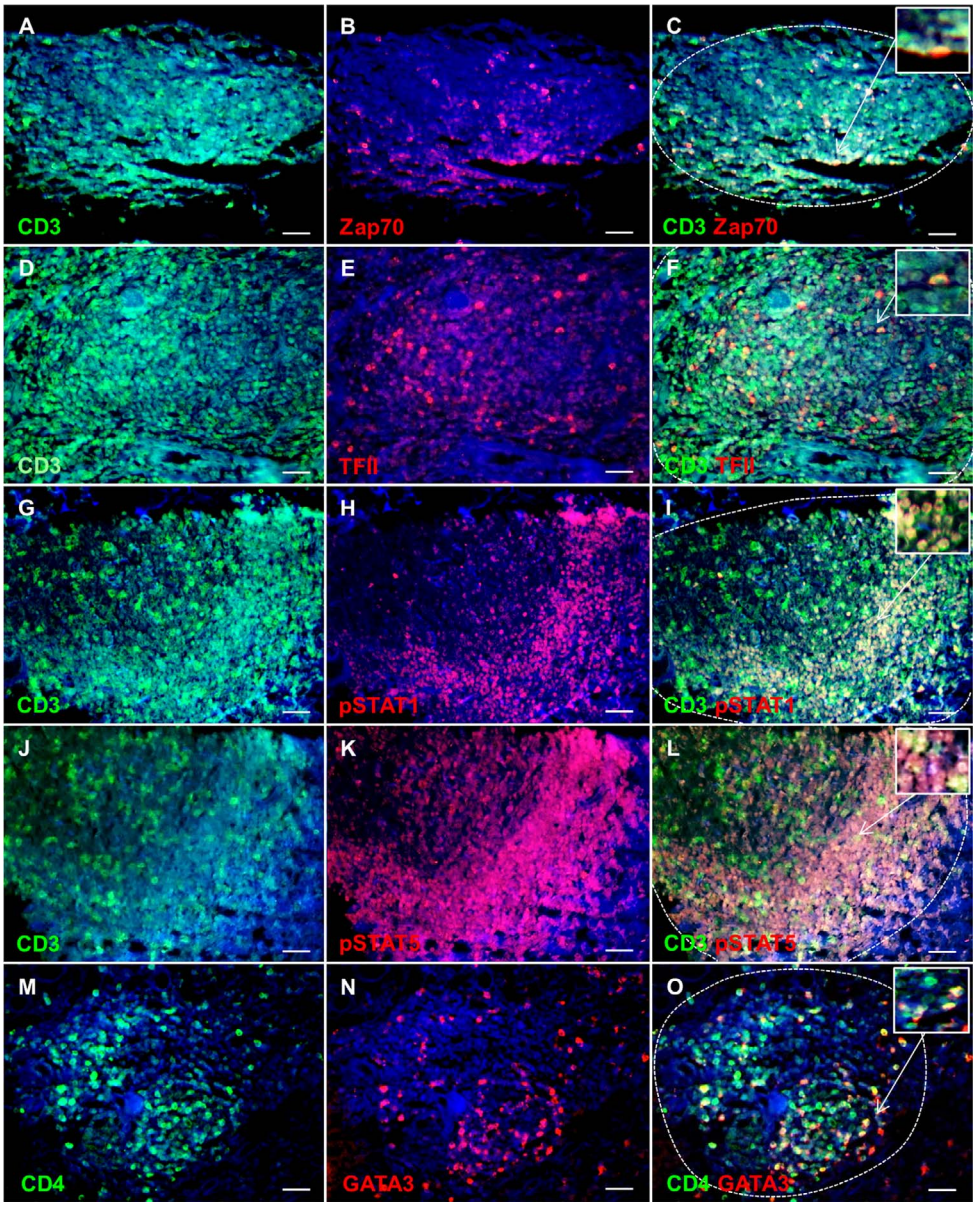

FIGURE 4. Intraocular lymphoid aggregates express immune cell transcription factors. (**A**) A subpopulation of $CD3^+$ T cells (*green*) coexpresses (**B**) Zap70 (red). (**D**) $CD3^+$ T cells (*green*). (**E**) Transcription-factor IIB (TFIIB, *red*) is mainly expressed by (**D**) T cells. (**G**) A large subpopulation of $CD3^+$ T cells (*green*) at the margin of the lymphoid aggregates is positive for (**H**) pSTAT1 (*red*). (**J**) More T cells (*green*) are positive for (**K**) pSTAT5 (*red*). (**N**) GATA3 (*red*) is mainly expressed by a subpopulation of (**M**) $CD4^+$ cells (*green*). (**C**, **F**, **I**, **L**, **O**) Merged pictures of respective single stainings, overlay results in *yellow color*. Cell nuclei are stained with DAPI (*blue*). Location of lymphoid follicles is marked with a *white dashed line*. *White scale bars*: Length of 20 μm (magnification: ×40). Mapped follicles are located in ciliary body.

For the first time to our knowledge, we could characterize the expression of different transcription factors associated with immune cell functions in the intraocular lymphoid follicles. We provided evidence for a scattered pattern of TFIIB-expressing T cells (Fig. 4F) in the ectopic lymphoid follicles of ERU cases. Transcription factor IIB is widely reported as an essential component for transcription initiation by RNA polymerase II in eukaryotic cells.[25] It also forms functional ARID3A DNA-binding complexes, activates immunoglobulin heavy-chain transcription upon B-lymphocyte activation, and, therefore, is linked to B-cell maturation.[26] Interestingly, in analyzed lymphoid-like follicles of ERU eyes, TFIIB expression could be detected only in T cells (Fig. 4F). Additionally, a clear proportion of B (Fig. 5I) and T cells (Fig. 5F) and few germinal center cells (Fig. 5L) coexpressed IRF4. In T cells, IRF4 expression is detectable in Th1, Th2, Th17, and Treg cells,[27] and involves the activation of NF-κB family members.[28] Additionally, IRF4 expression is upregulated by T-cell activation through TCR engagement[27] and various mitogenic stimuli.[28] The preferential expression of transcription factors that are typical for matured cells and the cellular composition and structural organization in these inflammation-associated ectopic structures indicated a high organization of adaptive immune responses in these aggregates in our point of view.

A further marker analyzed in this study was CD166, which was found consistently expressed on B (Fig. 5C) and T cells in tertiary lymphoid tissues in ERU cases (Fig. 5B). CD166 is encoded by the activated leukocyte cell adhesion molecule (*ALCAM*) gene and is a ligand for CD6. CD6 has a role in

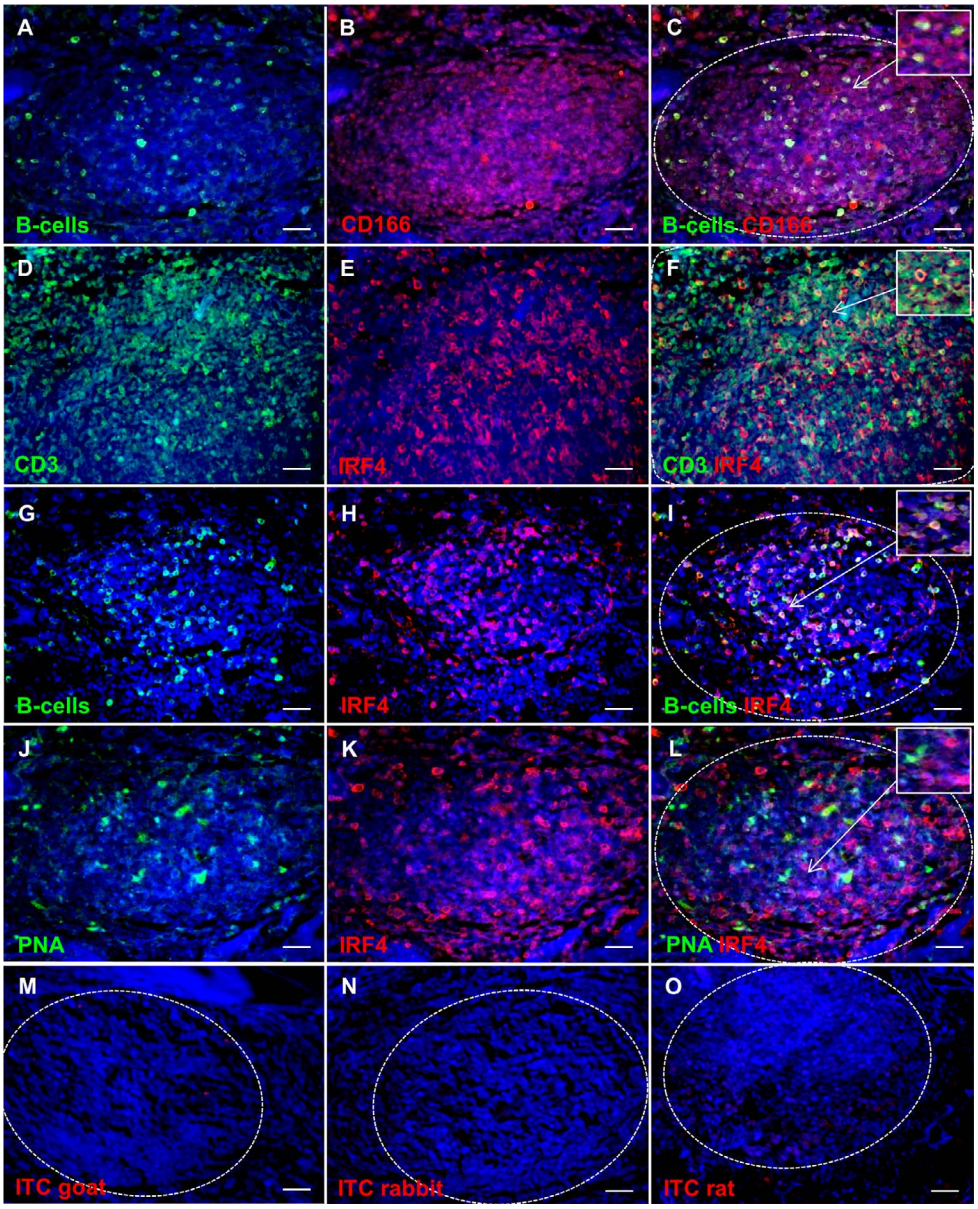

Figure 5. Further characterization of lymphoid-like follicles in spontaneous recurrent uveitis. (**A**) Sparse distribution of B cells (*green*). (**B**) Most cells in lymphoid aggregate are positive for CD166 (*red*). (**D**) A proportion of $CD3^+$ cells (*green*) and (**G**) of B cells (*green*), and (**J**) few PNA^+ cells (*green*) coexpress (**E**, **H**, **K**) IRF4 (*red*). (**C**, **F**, **I**, **L**) Merged pictures of respective single stainings, overlay results in *yellow color*. (**M**, **N**, **O**) Respective stainings of lymphoid-like follicles in ciliary body of ERU eyes with isotype controls (ITC) for (**M**) anti-goat IgG (*red*), (**N**) anti-rabbit IgG (*red*), and (**O**) anti-rat IgG (*red*) antibodies show no dyeing. Cell nuclei are stained with DAPI (*blue*). Location of lymphoid follicles is marked with a *white dashed line. White scale bars*: Length of 20 μm (magnification: ×40). Mapped follicles are located in ciliary body.

autoimmune diseases like rheumatoid arthritis or multiple sclerosis[29] and is expressed on activated T cells, B cells, and monocytes. CD166 was shown to facilitate $CD6^+$ leukocyte trafficking into the central nervous system in animal models.[30] Our finding could provide an interesting link to clarify the molecular mechanisms underlying the transmigration of lymphocytes into the ERU-diseased eyes, a key event of uveitis pathogenesis. Characterization of CD166 function in uveitis will be a subject of further investigation by us in the future.

Interestingly, we detected a prominent broadly striated expression of phosphorylated STAT1 and especially STAT5 in the periphery of the tertiary lymphoid structures (Figs. 4H, 4K), exclusively expressed in T cells (Figs. 4I, 4L). In contrast, the follicles were negative for pSTAT3 and pSTAT6 (data not shown). Activation of STAT proteins in general initiates signaling cascades of many cytokines and has a crucial role in the differentiation of Th cells.[31] This differentiation to various Th phenotypes is controlled by different STATs, through their activation by Th-specific cytokines. In our study, we found a high percentage of $pSTAT1^+$ and $pSTAT5^+$ T cells. It currently is unknown what kind of Th cells really are controlled by these STATs in horse immune cells, but in mice these STAT proteins are activated in response to IL-2 and IFN-γ, and are concomitant crucial mediators for inhibition of Th17 differentiation.[32] Additionally, STAT1 was shown to suppress Th17 differentiation via IL-27.[33] Interferon-γ and Th17 were detected in ERU cases.[10] Th17 is important in experimental autoimmune uveitis (EAU),[34] but uveitis also can be induced in $Th17^{-/-}$ mice.[35] Studies in $Th17^{-/-}$ mice showed, that IL-17 has an important role in later stages of uveitis and leads to a more

severe course of disease, but it is not a prerequisite of disease induction, where Th1 responses are important.[35] In retinal Mueller glial cells, we also found IFN-γ expression in context of ERU, what points to the involvement of a Th1-mediated response in ERU.[36] Currently, it is not fully elucidated if Th1 or Th17 response initially cause ERU, or if both immune responses are involved in the pathogenesis of this disease. Also, the importance of other Th subsets for the onset of the equine uveitis still is under consideration. Therefore, the meaning of Th1-associated transcription factor activation in the ectopic lymphoid follicles is interesting in our point of view. The antibody used here for detection of Tbet, the Th1 master transcription factor for Th1 cells, did not work in paraffin embedded sections, but we will try to identify the Th subsets besides the small proportion of identified Th2 cells (Fig. 4N, GATA3$^+$) in future experiments, when respective antibodies will be available.

In summary, the lymphoid-like follicles essentially consist of T cells, most of them CD4$^+$, with a scattered pattern of a few B cells (Fig. 3B; B cells, red) and PNA$^+$ germinal center cells. Analysis of transcription factors reveals existence of some Th2 cells (Fig. 4N; GATA3, red). Examinations on remaining Th subsets, like Th1, Th17, or Treg, could not be performed yet, because antibodies to associated master transcription factors Tbet, RORγT, and FoxP3 did not work in these paraffin embedded sections. However, the clear existence of pSTAT1$^+$ (Fig. 4H) and pSTAT5$^+$ (Fig. 4K) T cells points to an inhibition of the Th17 pathway.[32,33] Additionally, STAT5 acts as a positive regulator of Th1-, Th2-, and Treg-cell differentiation, whereas STAT1 is known to be especially activated in Th1 cells,[37] indicating a significant proportion of activated Th1 cells in the tertiary follicles. Altogether, our data point to the development of different Th subsets in lymphoid-like follicles in ERU eyes. Those informations are fundamental for understanding the pathomechanisms contributing to the onset of ERU and to the formation of the tertiary lymphoid follicles in particular, and, therefore, should be accomplished in future studies.

In patients with other autoimmune diseases, like Hashimoto's thyroiditis, Sjögren's syndrome, multiple sclerosis, and others, formation of ectopic lymphoid-like structures was dependent on disease-specific antigens.[38] Also in the R161H mouse, cells in the lymphocyte aggregates were specific for the retinal autoantigen IRBP, because the model is based on the expression of an IRBP-specific transgenic T-cell receptor.[9] We could not analyze antigen specificity of the intraocular lymphoid cells in situ in this study, but IRBP-immunized horses produced similar structures at the same sites.[18] Therefore, we hypothesized that the cells in the spontaneous ERU cases also are retinal autoantigen-specific and the follicles developed in response to these antigens for long-term maintenance of the immune response.

In contrast with the observations in R161H mice, where presence of lymphoid structures was detected in only 40% of cases and was correlated with lower clinical scores,[9] lymphoid follicle formation was detectable in most ERU cases and also in severe forms of disease.[21] This also was the case in IRBP-induced uveitis of horses.[18] We could examine only one point of time in the course of spontaneous uveitis, since eyes were mostly available at late stages of disease; therefore, we cannot clarify when the follicles exactly developed. The cellular composition of these highly organized tertiary lymphoid organs does not entirely clarify if these cells contribute more to inflammation or to attenuation of the intraocular immune response. In any case, autoaggressive T cells from peripheral blood invade the eye immediately before an uveitic attack, as we have shown earlier in induced models of horse uveitis[18,17] and in spontaneous ERU.[39] This points to a significant role of these peripheral lymphocytes for the intraocular inflammation.

Ectopic lymphoid structures also were reported from target organs of other autoimmune diseases, for example, in the brains of patients with multiple sclerosis[38,40] or in pancreatic islets of mouse diabetes models.[41,42] The infiltrates can just form diffuse lymphocyte aggregates or highly organized and sophisticated structures.[38] The presence of ectopic lymphoid structures correlated with a poor outcome in autoimmune diseases, like rheumatoid arthritis,[43] but with a milder disease course in experimental uveitis in mice.[9] In our analyses, the follicles were associated with more severe disease courses of experimental[18] and spontaneous uveitis of horses.[21] To date, it is largely unknown what governs the formation of tertiary lymphoid structures in certain individuals/diseases[44] and in particular its relevance for the course of recurrent uveitis. The finding of highly organized ectopic lymphoid tissue in a naturally occurring disease model underscores the significance of these structures in chronic autoimmune inflammation of the inner eye, and the mechanisms contributing to their formation, maintenance, and function should be further investigated.

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