Journal of Neuroimmunology 293 (2016) 114–122

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Systemic ocular antigen immunization leads only to a minor secondary immune response

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ARTICLE INFO

Article history:
Received 9 December 2015
Received in revised form 24 February 2016
Accepted 26 February 2016

Keywords:
Glaucoma
Retina degeneration
Lymphocytes
Granulocytes
Macrophages
FACS

ABSTRACT

The immunization with optic nerve homogenate antigen (ONA) or S100 induced retinal degeneration. Since many neurological diseases are reinforced or initiated by immune cells, leucocytes were analyzed. $CD3^+$ T-cells in the retina increased slightly in ONA rats, but not in S100 treated retinas. No $CD45R^+$ B-cells and granulocytes could be detected in the retinas. At early stages, $CD3^+$ cells, $Iba1^+$ macrophages and granulocytes of the secondary lymphoid organs were not affected. Yet, the sole injection of pertussis toxin led to a shift to fewer $CD45R^+$ cells and more granulocytes in spleens. Later, splenic $Iba1^+$ macrophages were increased in both groups. We conclude that the retinal infiltration of lymphocytes is not crucial for the degeneration process and rather an epiphenomenon.

1. Introduction

Neurological diseases lead to degenerating effects, which are often accompanied by an autoimmune response, especially by microglia and lymphocytes (Hendrickx et al., 2014; Saunders et al., 2012; Zotova et al., 2013).

Glaucoma, a neuronal disease, is based on the loss of the retinal ganglion cells (RGCs) and optic nerve fibers (Almasieh et al., 2012; Vrabec and Levin, 2007). The main cause is still unidentified, but the immune system seems to be involved in the pathogenesis (Gramlich et al., 2013; Joachim et al., 2007; Yang et al., 2001). It is known that a systemic immunization with the ocular antigens, ONA or S100, causes degenerative effects on the RGCs (Casola et al., 2015; Laspas et al., 2011). ONA is a bovine optic nerve homogenate antigen. S100B, which is a compound of the ONA, is a calcium binding protein and is mainly expressed by glia cells (Huttunen et al., 2000). It is a potent mediator of neuronal cell death through inflammation and oxidative stress (Rothermundt et al., 2003), it also has an effect on glial subtypes (Bianchi et al., 2007; Villarreal et al., 2014). An altered antibody titer against S100B was found in tear fluids of glaucoma patients (Grus et al., 2010), which led us to the assumption that the S100 protein plays a potential role in the pathogenesis of glaucoma. Interestingly, the immunization with ONA or S100B induces an early microglia response before the RGCs degenerated (Noristani et al., 2016). Furthermore, the cell death is accompanied by an increase of auto-antibodies (Joachim et al., 2014).

Now the question arises, whether lymphocytes are involved in the degenerative processes. B-cells seem to be a part of the process, since they were detected in the retina of glaucoma patients (Gramlich et al., 2013). Also, antibody deposits, produced by B-cells, were observed in the retinas of ONA immunized animals (Joachim et al., 2014; Joachim et al., 2013). Additionally, some evidences for the activation of T-cells were found in blood samples of glaucoma patients (Huang et al., 2010; Yang et al., 2001), in the human retina (Gramlich et al., 2013) and in the retina of a glaucoma animal model (Wax et al., 2008). All these findings implicate an involvement of lymphocytes in the pathogenesis of retinal degeneration. Lymphocyte activation and their proliferation occur in the secondary lymphoid organs, like spleen and cervical lymph nodes (cLN). Especially the cLN are involved in the lymphocyte reaction against CNS and retinal antigens, because both tissues have no conventional lymph system (Weller et al., 2010).

The aim of this project was to investigate the role of lymphocytes for the development of the retinal degeneration in an experimental autoimmune glaucoma model. Therefore, the retinas and the secondary lymphoid organs were examined at an early and later stage of this model.

Abbreviations: ONA, Optic nerve homogenate antigen; Co, Control; RGCs, Retinal ganglion cells; FA, Freund's Adjuvants; PTx, Pertussis toxin; IgG, Immunoglobulin G; CNS, Central nervous system; cLN, Cervical lymph nodes.

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http://dx.doi.org/10.1016/j.jneuroim.2016.02.017
0165-5728/

2. Material and method

2.1. Animals

Male Lewis rats from Charles River (176–200 g; Sulzfeld, Germany) were used in this study. The experiments were carried out in conformity with the ARVO statement for the use of animals in ophthalmic and vision research. The study was approved by the animal care committee of North Rhine-Westphalia (Germany). The rats were housed under environmentally controlled conditions with free access to chow and water. Detailed observations of possible neurological deficits and eye exams were performed regularly.

2.2. Immunization

The bovine optic nerve homogenate antigen (ONA) preparation was already described in previous studies (Joachim et al., 2014). The purified antigen, S100 (=S100B, Sigma-Aldrich, order no: S6677), is commercially available. 1 ml of the ONA (8 mg/ml) or 200 μl of the S100B solution (1 mg/ml) was mixed separately with Freund's Adjuvants (FA; 500 μl in ONA, 200 μl in S100, Sigma-Aldrich) and 3 μg Pertussis Toxin (PTx; Sigma-Aldrich). Both mixtures were administered intraperitoneally. The control group (Co) received 1 ml sodium chloride (NaCl) mixed with 500 μl FA and 3 μg PTx and the Naïve group was sham immunized only with 1.62 ml NaCl. The rats were sacrificed after 14 and 28 days.

2.3. Spleen dimension measurement

After extraction of the spleens at days 14 and 28, they were weighed and the dimensions (length [L], width [W] and height [H]) were measured with an electronic measuring caliper (Promat). The spleen volume was calculated using the formula for a prolate ellipsoid: $V = \frac{\pi}{6} \times L \times W \times H$. This is an established formula for the volume determination of solid organs, like the human spleen (Yetter et al., 2003).

2.4. Western blot

One retina per rat was used for Western blot analyses (n = 4–5/group). The retinal proteins were isolated by mechanical and chemical methods. The frozen retinas were homogenized with a metal homogenizer (Neolab), chemically treated with 150 μl of a commercial lysis buffer (RIPA buffer, Cell signaling technology) and a protease inhibitory solution (Sigma-Aldrich) and mechanically grinded with ultrasound. The cell components were separated with a centrifuge for 30 min. The protein concentration was determined with a commercial bicinchoninic acid assay (BCA). 20 μg per sample were loaded on a lane of a 12% Bis-Tris gel (NuPAGE, Invitrogen). After the blotting step with the NuPAGE transfer buffer (Invitrogen), the nitrocellulose membranes were blocked with a mixture of 5% milk powder in a PBS/0.05% Tween-20 solution. Brn-3a (1:200, Santa-Cruz, order no: sc-31,984) was stained with donkey anti-rabbit Alexa Fluor 680 (1:5000, Invitrogen, order no: A21088) and β-actin (mouse, 1:5000, Sigma Aldrich, order no: A2228) was used with IRDye donkey anti-mouse DL800 (1:15.000, LICOR, order no: 926-32212). The protein bands were recorded and analyzed with the Odyssey infrared imager system 2.1 (LI-COR Bioscience). The relation between Brn-3a and β-actin signal intensity was measured.

2.5. Preparation of cell suspension

After 14 days, blood (n = 5-6/group), cervical lymph nodes (cLN, n = 7/group), spleens (n = 6-7/group) and eyes (n = 7/group) were obtained. A single cell solution of the four tissues was prepared with the following methods.

2.5.1. Blood

6 ml blood was taken by a cardiac puncture and collected in 15 ml tubes filled with EDTA (1 mg/ml blood), a coagulation inhibitor. The mononuclear cells in the blood were separated with Histopaque 1083 (Sigma-Aldrich) according to the manufactures instructions. The mononuclear cells were separated in the Histopaque interface layer and the granulocytes in the upper Histopaque 1083 layer. Both solutions were transferred into a 15 ml centrifuge tube. The cell solution was cleaned from the remaining Histopaque 1083 with PBS and centrifuged. At the end, the cell pellet was resolved in 5 ml of a "wash solution" (PBS/3% FCS/1% EDTA 0.2 M).

2.5.2. Cervical lymph nodes and spleen

A single cell solution of the cLN and the spleen was prepared by pushing the tissues through a cell strainer (cLN = 40 μm; spleen = 70 μm; BD Falcon) with a syringe plunger in a 50 ml centrifuge tube. A PBS/10% FCS solution was added to the tubes of both cell solutions and centrifuged. The supernatants were discarded and the cell pellet of the cLN was resuspended with 4 ml "wash solution". The suspensions were resolved with 15 ml aqua dest. (lysis of erythrocytes) and then washed with PBS/10% FCS. Then, the cell pellets were resuspended with 20 ml "wash solution".

2.5.3. Retina

A modified protocol of Copland et al. (Copland et al., 2008) and Luger et al. (Luger et al., 2008) was used. The left and the right eye were enucleated and both retinas were pooled in 1 ml of RPMI/10% FCS medium. Both retinas were dispersed by a vigorous pipetting. 6 ml medium was added and the tube was centrifuged. The cell pellet was resuspended with 3 ml of an enzyme solution (1 mg Collagenase I and 0.2 mg DNase in RPMI/10% FCS medium) and incubated at 37 °C for 60 min. Then, the samples were dispersed by pipetting several times, washed, filtered (40 μm) and suspended in a 1 ml "wash solution".

2.6. Flow cytometry

The cell count of the four suspensions (blood, cLN, spleen and retina) was determined with trypan blue (diluted 1:2) in an improved Neubauer counting chamber (Assistant). $1x10^5$ cells were placed in a 1.5 ml tube (Sarstedt). At first, the Fc_γ receptors (CD32, BD biosciences, order no: 550,271) were blocked (10 min). Then, the cells were washed and double stained with CD3-FITC (2.5 μg, T-cells; eBioscience, order no: 11-0030) and CD45R-PE (0.2 μg, B-cells; eBioscience, order no: 12-0460) or their isotype controls mouse IgG3,κ-FITC (2.5 μg; eBioscience, order no: 11-4742) and mouse IgG2b,κ-PE (0.2 μg; eBioscience, order no: 12-4732) for 30 min. The granulocytes were stained separately with the anti-Granulocyte marker-FITC (Clone HIS48, 0.25 μg; eBioscience, order no: 11-0570) or the isotype control, mouse, IgM,κ-FITC (0.25 μg; eBioscience, order no: 553,474), for 30 min. The cells were washed and resuspended with 1.5% paraformaldehyde (PFA; Merck). The cells were counted with a Cyflow FACS (Partec) and the analysis was performed with FloMax 2.7 (Partec). The autofluorescence signal was subtracted by gating on the isotype control. The histogram overlay was displayed with Flowing Software 2.5.1 (University of Turku, Finland). The gate was placed on the isotype control histogram.

2.7. Immunohistochemistry

Eyes, optic nerves and spleens were fixed in 4% PFA for 60 min and then treated with 30% sucrose overnight. The cLN remained untreated. The tissues were embedded in NEG-50 Tissue-Tek medium (Thermo Fisher). Cryo cross-sections (eye = 10 μm, optic nerve = 4 μm, spleen = 5 μm and cLN = 8 μm) were cut with a microtome (Thermo Fisher), mounted on slides and fixed in ice cold aceton for 10 min. Brn-3a was used to label the RGCs of the retina (1:100, Santa-Cruz, order no: sc-