Molecular Immunology 90 (2017) 255–263

Contents lists available at ScienceDirect

Molecular Immunology

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

Elevated intrathymic sphingosine-1-phosphate promotes thymus involution during sepsis

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ARTICLE INFO

Keywords:
Sepsis
Thymus involution
Sphingosine-1-phosphate
T cells
Apoptosis

ABSTRACT

Sepsis mouse models revealed thymus atrophy, characterised by decreased thymus weight and loss of thymocytes due to apoptosis. Mice suffered from lymphopenia, a lack of T cells in the periphery, which attenuates their ability to fight against recurring and secondary infections during sepsis progression. Key players in thymus atrophy are IL-6, which is directly involved in thymus involution, and the sphingosine-1-phosphate – sphingosine-1-phosphate receptor 1 signaling, influencing thymocytes emigration. In healthy individuals a sphingosine-1-phosphate (S1P) gradient from lymphoid organs to the circulatory system serves as signal for mature T cell egress. In the present study we investigated, whether inhibition of S1P generation improves thymus involution. In sepsis, induced by cecal ligation and puncture (CLP), S1P in the thymus increased, while it decreased in serum, thus disrupting the naturally occurring S1P gradient. As a potential source of S1P we identified increased numbers of apoptotic cells in the thymic cortex of septic mice. Pharmacological inhibition of the S1P generating sphingosine kinases, by 4- [[4-(4-Chlorophenyl)-2-thiazolyl]amino]phenol (SK I–II), administered directly following CLP, prevented thymus atrophy. This was reflected by lymphocytosis, diminished apoptosis, decreased IL-6 expression, and an unaltered thymus weight. In addition SK I-II-treatment preserved the S1P balance and prevented S1P-dependent internalization of the sphingosine-1-phosphate receptor 1. Our data suggest that inhibition of sphingosine kinase and thus, S1P generation during sepsis restores thymic T cell egress, which might improve septic outcome.

1. Introduction

Sepsis is a highly complex, life-threatening disease (Engel, 2007), characterised by pro- and anti-inflammatory responses resulting in immune paralysis. The hypo-responsive phase, characterised by a massive T cell loss in the periphery, contributes to impaired functions of the adaptive immune system (Bone, 1996). The breakdown of the complex immune cell network results in increased susceptibility to recurrent and secondary infections, often leading to patient death (Hotchkiss et al., 2013). T cell loss during the anti-inflammatory phase is evoked by e.g. PPARγ-dependent apoptosis (Schmidt et al., 2011; Soller et al., 2006). Thymus involution decreasing T cell emigration also reduces T cell counts in blood (Lynch et al., 2009). Acute thymus involution is caused by stressors such as hypoxia, trauma, malnutrition, and infections (Dooley and Liston, 2012; Savino et al., 2007). Contrary to age-dependent involution, acute involution is transient and reversible. Two distinct mechanisms can be responsible for thymus involution; an infection-sensing programmed involution triggered by thymic epithelial cells (TECs) and an inflammation-mediated mechanism induced by glucocorticoids, or stress-stimuli such as IL-6, inducing thymic atrophy through promotion of apoptosis in thymocytes (Dooley and Liston, 2012). The involution reduces tissue mass, due to an increased perivascular space, a prominent loss of thymocytes, and a decreased output of naive T cells, thereby disrupting the T cell balance (Gui et al., 2012; Lynch et al., 2009). This status contributes to a reduced immune surveillance and a higher vulnerability to infections (Gruver and Sempowski, 2008; Ponnappan and Ponnappan, 2011). The emigration of T cells from the thymus is orchestrated by a sphingosine-

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http://dx.doi.org/10.1016/j.molimm.2017.08.011
Received 21 February 2017; Received in revised form 3 July 2017; Accepted 14 August 2017

1-phosphate (S1P) gradient. Naive T cells express the S1P receptor 1 (S1PR1) on their surface in order to follow the S1P gradient from lymphoid organs to the circulation (Matloubian et al., 2004). Exposed to high S1P in blood or lymph, S1PR1 is internalized and T cells are retained in the circulation (Lo et al., 2005). Additionally, S1P binds to S1PR1 on sinus-lining endothelial cells, promoting the closure of junctional contacts and preventing thymocyte respectively T cell egress (Wei et al., 2005). S1P mediates a variety of biological responses by promoting proliferation, stimulating survival, and preventing apoptosis (Liu et al., 2003; Olivera et al., 1999; Spiegel et al., 1998; Weigert et al., 2006). The phosphorylation of sphingosine to S1P is catalyzed by sphingosine kinases (SphK). Two mammalian SphK isoforms were described, SphK1 and SphK2 (Maceyka et al., 2005). The steady-state level of S1P is regulated through its formation by kinases and degradation by S1P lyase and phosphatases (Changyong Li et al., 2009). S1P is generated by various cell types (Olivera et al., 2010), moreover apoptotic cells are known to release increased amounts of S1P (Weigert et al., 2006). Here, we investigated the role of S1P in sepsis–induced thymus involution. Our findings provide evidence that thymus involution results from a disrupted S1P balance. Apoptotic thymocytes generate increasing amounts of S1P and prevent T cell egress towards the periphery. S1P itself elevates IL-6, which in turn accounts for thymus atrophy. The pharmacological inhibition of SphK reduced thymus involution during sepsis progression.

2. Material and methods

2.1. Reagents

50 mg/kg 4-[[4-(4-Chlorophenyl)-2-thiazolyl]amino]phenol (SK I–II) from Cayman Chemicals (Ann Arbor, USA) was used for pharmacological inhibition of sphingosine kinases in mice.

2.2. Animals

For sepsis experiments, we used 8–12 weeks old male C57BL/6 mice or $Sphk1^{tm1Npa}$ resp. $Sphk2^{tm1Npa}$ mice (kindly provided by Novartis (Vienna, Austria)). Animals were maintained under artificial day-night cycles (12 h light-dark cycles) and received a standard mouse diet and water *ad libitum*. Animals are kept in groups, up to five animals in type-II long or comparable individually ventilated cages (IVC) with nest material as enrichment. Animal experiments followed the guidelines of the Hessian animal care and use committee (approval no. F144-06 and FU/1042).

2.3. Animal procedures

The cecal ligation and puncture model (CLP) was performed as previously described (Rittirsch et al., 2008). Mice were anesthetized with Ketamine/Xylazine. The cecum was punctured once through-and-through with a 20-gauge needle. Sham mice were treated the same way but without ligation and puncture of the cecum. Inhibitor treated mice received 50 mg/kg SK I-II *i.p.* (solved in DMSO/EtOH) directly following CLP surgery. Mice were sacrificed 24 h or 48 h after CLP by cervical dislocation. Thymus and spleen were dissected. Blood was isolated. Samples were kept on ice prior to experiments.

Animals received buprenorphine (0,05 mg/kg) directly following the surgery and every 6- 8 h. Animals were scored at the predetermined time points (3, 8, 21 and 30 h after the surgery). The clinical severity score reflects behavior, weight, temperature, mobility, eating behavior and appearance. Humane endpoints were used during the animal survival study, animals were euthanized when they met criteria determinate in an Animal Health Score Sheet.

2.4. Cell isolation

Thymus weight was determined. Organs (thymus and spleen) were sliced and approximately 10 mg tissue was homogenized in PBS (supplemented with 0.5 % BSA and 2 mM EDTA) with a dounce homogenizer to obtain a cell suspension.

2.5. Flow cytometry

For analysis of blood cells, 100 µL blood was stained followed by erythrocyte lysis (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). An aliquot of the cell suspension was first incubated with purified anti-mouse CD16/32 (BioLegend, San Diego, CA, USA) for 15 min on ice, to block non-specific antibody binding to Fc receptors. For detection of CD3, CD45, CD19, CD4, CD8, CD62L, CD44 and S1PR1 cells were incubated with anti-CD3e-PE-CF594, anti-CD45-V500, anti-CD4-Pacific Blue, anti-CD44-AF700 (all BD Biosciences, Heidelberg, Germany), anti-CD8a-Brilliant Violet 650, anti-CD3e-AF488 (BioLegend), anti-CD19-FITC (ImmunoTools, Friesoythe, Germany), anti-CD62L-PE (USBiologicals, Salem, MA, USA) and anti-mEDG-1/S1P1-PE (R & D Systems). CountBright™ Absolute Counting Beads (Life Technologies, Carlsbad, USA) were used to determine the absolute number of CD4 and CD8 positive cells. For annexin V staining an aliquot of cells was incubated with anti-annexin V FITC (ImmunoTools) in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.5). Samples were acquired with a LSRII/Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software 7.6.1 (Treestar, Ashland, OR, USA).

2.6. Immunohistochemistry

Tissue samples were dehydrated in alcohol (Sigma-Aldrich) and embedded in paraffin. Sections of 4 µm were deparaffinized in xylene (Sigma-Aldrich), rehydrated in alcohol. For antigen retrieval, the sections were boiled for 12 min in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Endogenous avidin and biotin was blocked by using a biotin-blocking system (DAKO, Hamburg, Germany). For stainings, a Catalyzed Signal Amplification (CSA) kit (DAKO) was used according to manufactureŕs instructions. The primary antibody anti-cleaved caspase 3 (Cell Signaling, Danvers, MA, USA) was incubated at 4 °C overnight. Afterwards, the corresponding biotinylated secondary antibody (biotinylated anti-rabbit from Axxora, Loerrach, Germany) was applied. For color development diaminobenzidine (DAB) (contained in the CSA kit) was added as a substrate to the peroxidase-coupled secondary antibody. Sections were counter stained with Mayeŕs hemalum solution (Merck, Darmstadt, Germany) and mounted with Aquatex (Merck, Darmstadt, Germany). Slides were monitored with the Axioskop 40 (ZEISS, Goettingen, Germany) equipped with the AxioVision Release 4.8.2 software (10 x magnification/N.A. value 0.25)

2.7. Quantitative PCR

Total RNA from thymocytes was isolated using PeqGold RNAPure Kit (PeqLab Biotechnologie GmbH, Erlangen, Germany) as described by the manufacturer. Reverse transcription was done with 2 µg of total RNA using iScript™ cDNA Synthesis kit (Bio-Rad GmbH, Munich, Germany). Quantitative PCR (qPCR) was performed with IQ SYBR® Green Supermix (BioRad) according to the manufacturer's protocol. The following primers were used (primer sequences and accession no. in Table 1): 18 S, SPHK1, EDG-1 (S1PR1). QuantiTect® Primer Assays from Qiagen (Hilden, Germany) were used for determination of CD69, KLF2, IL6 (assay name and accession no. are provided in Table 2). Values were normalized to 18S ribosomal RNA.