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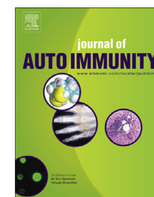

Deletion of WASp and N-WASp in B cells cripples the germinal center response and results in production of IgM autoantibodies

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ABSTRACT

Humoral immunodeficiency caused by mutations in the Wiskott-Aldrich syndrome protein (WASp) is associated with failure to respond to common pathogens and high frequency of autoimmunity. Here we addressed the question how deficiency in WASp and the homologous protein N-WASp skews the immune response towards autoreactivity. Mice devoid of WASp or both WASp and N-WASp in B cells formed germinal center to increased load of apoptotic cells as a source of autoantigens. However, the germinal centers showed abolished polarity and B cells retained longer and proliferated less in the germinal centers. While WASp-deficient mice had high titers of autoreactive IgG, B cells devoid of both WASp and N-WASp produced mainly IgM autoantibodies with broad reactivity to autoantigens. Moreover, B cells lacking both WASp and N-WASp induced somatic hypermutation at reduced frequency. Despite this, IgG1-expressing B cells devoid of WASp and N-WASp acquired a specific high affinity mutation, implying an increased BCR signaling threshold for selection in germinal centers. Our data provides evidence for that N-WASp expression alone drives WASp-deficient B cells towards autoimmunity.

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1. Introduction

Humoral immunity generates long-lived plasma cells and memory cells and depends on B cell affinity maturation in germinal centers (GCs). The GC is organized into two spatially separated zones. The light zone (LZ) of the GC contains the network of follicular dendritic cells (FDCs) that endocytose and recycle antigen to facilitate B cell affinity maturation [1]. The dark zone (DZ) consists of highly proliferating B cells that express activation induced deaminase (AID) to induce somatic hypermutation (SHM) and immunoglobulin (Ig) class switch recombination [2]. Newly mutated and Ig class switched B cells migrate from the DZ to test their BCR for antigen recognition in the LZ on antigen-covered FDCs and compete for help from antigen-specific T follicular helper (T_{FH}) cells [3,4]. The affinity of the B cell receptor (BCR) for the antigen on FDCs is proportional to antigen uptake and frequency of MHC class II – peptide presentation to T_{FH} cells [5]. Therefore, B cells that express BCR with high affinity for antigen out-competes B cells with low affinity BCR and only high affinity B cells develop into plasma cells and memory cells [4,5]. B cell migration between the DZ and LZ is mediated by the chemokine receptors CXCR4 and CXCR5 [6] and both interzonal migration [3] and intrazonal migration [7] drives SHM in the DZ and selection of B cells in the LZ.

Extensive cell migration, cell-to-cell communication, and cell division during the GC response critically rely on BCR signaling and the dynamics of the actin cytoskeleton. Signaling from the BCR to the actin cytoskeleton is regulated by the intracellular signaling

Abbreviations: AID, activation induced deaminase; BCR, B cell receptor; C_H, constant heavy chain; DZ, dark zone; FDCs, follicular dendritic cells; GC, germinal center; GEF, guanine exchange factor; Ig, immunoglobulin; LZ, light zone; MZ, marginal zone; NP-KLH, 4-Hydroxy-3-nitrophenylacetyl Keyhole Limpet Hemocyanin; N-WASp, neuronal Wiskott-Aldrich syndrome protein; SHM, somatic hypermutation; T_{FH}, T follicular helper; V_L, variable light chain; V_H, variable heavy chain; WAS, Wiskott-Aldrich syndrome; WASp, WAS protein.

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axis of guanine exchange factors (GEFs, including Vav1, Vav2, and Dock8), that activate Rho GTPases (such as Cdc42, Rac1, and Rac2), that in turn activate WASp family members (such as WASp and N-WASp) [8,9]. While WASp is exclusively expressed in hematopoietic cells [10,11], the homologous protein N-WASp is ubiquitously expressed [12] and complete deletion of N-WASp is embryonic lethal [13]. Deletion of both WASp and N-WASp specifically in B or T cells markedly reduces B and T cell development and activation, implying that N-WASp can partially compensate for WASp deficiency [14,15]. Up to 70% of WAS patients develop autoimmune disease [16–18] and this has been attributed to defective suppression by WASp$^{-/-}$ T regulatory cells [19–22] and to intrinsic B cell dysfunction [23–25]. Moreover, three recent papers describe decreased BCR repertoire diversity in WAS patients associated with high production of autoantibodies [26–28]. Deletion of both WASp and N-WASp in B cells leads to absence of marginal zone (MZ) B cells associated with abolished IgG antibody responses to T-cell independent antigen [14]. Liu et al. recently showed that B cell specific deletion of N-WASp enhances and prolongs BCR signaling, suggesting that N-WASp is a critical negative regulator of B cell activation [29]. The exact function of WASp and N-WASp in the GC response and in regulation of peripheral B cell tolerance remains largely unknown.

Here we sought to define the role of WASp and N-WASp in the GC response to autoantigens and non-self antigen. To accomplish this, we used mice lacking WASp (WASp$^{-/-}$ mice) and mice lacking WASp and N-WASp in B cells (WASp$^{-/-}$N-WASp$^{fl/fl}$CD19$^{Cre/+}$ mice). We show that WASp$^{-/-}$ and WASp$^{-/-}$N-WASp$^{fl/fl}$CD19$^{Cre/+}$ mice formed GCs in response to apoptotic cells as a source of autoantigens, however, the GCs had reduced polarization into DZ and LZ and B cell retained longer and proliferated less in GCs. WASp$^{-/-}$ mice had elevated titers of autoreactive IgG antibodies and generated high affinity IgG antibodies. In contrast, WASp$^{-/-}$N-WASp$^{fl/fl}$CD19$^{Cre/+}$ produced mainly IgM autoantibodies with broad reactivity to autoantigens and failed to induce increased titers of high affinity IgG antibodies. Our results suggest that N-WASp expression alone drives WASp-deficient B cells towards autoimmunity.

2. Material and methods

2.1. Mice and BM transfer

Mice were housed at the animal facility of the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet under specific pathogen-free conditions. Animal experiments were carried out after approval and in accordance with guidelines from the local ethical committee (North Stockholm district court). Wildtype, WASp$^{-/-}$, and WASp$^{-/-}$N-WASp$^{fl/fl}$CD19$^{Cre/+}$ mice were littermates from mixed allele breedings of WT 129Sv mice, WASp$^{-/-}$ mice on a 129Sv background, N-WASp$^{fl/fl}$ mice on 129Sv background, and CD19$^{Cre/+}$ mice on C57Bl/6 background. For generation of mixed bone marrow (BM) chimeras, 2×10^7 total BM cells containing WASp$^{-/-}$ or WASp$^{-/-}$N-WASp$^{fl/fl}$CD19$^{Cre/+}$ BM (expressing CD45.2) with wildtype BM (expressing CD45.1) at a 3:1 ratio were transplanted via intravenous injection into lethally irradiated (13 Gy) wildtype 129Sv recipient animals.

2.2. Apoptotic cell immunization and anti-DNA ELISA

The apoptotic cells were prepared from syngeneic thymocytes cultured for 6 h in complete RPMI 1640 and 1 μM dexamethasone (Sigma–Aldrich). Age- and sex-matched littermate mice were injected intravenously weekly for 4 weeks with 10^7 apoptotic. Serum samples were collected weekly from the tail artery and anti-DNA autoantibodies were measured by ELISA [30,31]. In brief, anti-DNA titers of IgM and IgG were measured by ELISA using methylated BSA plus calf thymus DNA (Sigma–Aldrich) or total histone solution (Sigma–Aldrich) as capture. AP-conjugated secondary anti-IgM and -IgG antibodies (SouthernBiotech) were used for detection. As standard for analysis of serum from mice immunized with apoptotic cells, serial dilutions of a pool of serums from all samples was used. As standard for analysis of serum from 8 to 10 months old unchallenged mice, serial dilutions of an ANA positve B6.MRL-lpr or NZM2410 serum pool was used, or serial dilutions of mouse anti-human nucleosome IgG (BDbiosciences). All samples were tested in duplicates and corrected for background binding. Titration curves for IgM are shown in Supplementary Figure S1.

2.3. Labeling of apoptotic cells, immunohistochemistry and measurement in picture

Apoptotic cells were labeled with CFSE (Invitrogen) and analyzed by immunohistochemistry [32]. The following reagents were used: anti- B220 (RA3-6B2), -IgG1 (RMG1-1, BioLegend); -CD169 (MOMA-1; AbCam); -CD11c (HL3), -IgM (II/41), -CD35 (8C12; BD Biosciences), biotinylated peanut agglutinin (Vector Laboratories); goat anti-rat-AlexaFluor488, Strepavidin-AlexaFluor555 (Invitrogen). Images were collected with a Leica DM IRBE confocal laser scanning microscope (Leica Microsystems) equipped with 1 argon and 2 HeNe lasers, using an HC PL APO lens at 10x/0.40 CS and 90% glycerol (MP Biomedicals) and processed with Adobe Photoshop CS5 (Adobe Systems). The areas of GCs (PNA$^+$), of follicular (B220$^+$ cells surrounded by CD169$^+$ cells) and areas of CD35$^+$ in GCs and B220$^+$ area (excluding MZ) were measured on three images from each mouse of random sections using Fiji, ImageJ software (National Institutes of Health), and the ratio was calculated.

2.4. Autoantibody arrays

Serum from naïve mice and mice immunized with apoptotic cells were screened for reactivity to 95 different autoantigens using autoantibody array (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility) [33]. The array was probed with serum samples, developed with Cy3-labeled anti-IgG and Cy5-labeled anti-IgM, and then scanned at 635 nm and 570 nm fluorescence, respectively. Data were visualized by using MultiExperimentViewer, Cluster 3.0 (Open Source software available at http://mev-tm4.sourceforge.net).

2.5. DC isolation and flow cytometry

For DC isolation, spleens were cut up into small fragments and incubated at 37 °C for 1 h in 1 mg/ml collagenase III (Worthington) and 120 μg/ml DNAse (Roche) in RPMI-1640 medium. For flow cytometry, data was acquired on a FACSFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo Version 10 software for PC (TreeStar). The following antibodies were used: anti-V$_L$λ (R26-46), -CD62L (Mel14), -IgG1, -CD8 (53–6.7), -CD11b (M1/70), -CXCR4 (2BII/CXCR4), -CD44 (IM7), -CXCR5 (2G8), -GL7 (GL7), -CD95 (Jo2) (all BD Biosciences), -CD44 (eBioscience), Strepavidin-AlexaFluor555, LIVE/DEAD® (Invitrogen), -DEC205 (NLDC-145, Dendritics), -PD1 (RMP1-30), -CD3 (145-2C11), -CD4 (RM4-5), -CD11c (N418), -CD83 (Michel-19), -33D1 (33D1), -B220 (RA3-6B2), -CD138 (281–2), -IgM (RMM-1), Streptavidin-APC-Cy7 (all BioLegend), -NP8, -NP24 (all Biosearch Technologies) and -IgG1 (SouthernBiotech).

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