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IL10 restrains autoreactive B cells in transgenic mice expressing inactive RAG1

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Abstract

IL10 plays a dual role in supporting humoral immunity and inhibiting inflammatory conditions. B cells producing IL10 are thought to play a key regulatory role in maintaining self-tolerance and suppressing excessive inflammation during autoimmune and infectious diseases, primarily by inhibiting associated T cell responses. The extent to which B cells, through the provision of IL10, might function to sustain or inhibit autoantibody production is less clear. We previously described transgenic mice expressing catalytically inactive RAG1 (dnRAG1 mice), which show expansion of an IL10-compentent CD5⁺ B cell subset that phenotypically resembles B10 B cells, hypogammaglobulinemia, and a restricted B cell receptor repertoire with features indicative of impaired B cell receptor editing. We show here that B10-like B cells in dnRAG1 mice bind the membrane-associated autoantigen phosphatidylcholine (PtC), and that *in vitro* lipopolysaccharide (LPS) stimulation of dnRAG1 splenocytes induces a robust IgM response enriched in reactivity toward lupus-associated autoantigens. This outcome was correlated with detection of sIgM^{hi} B cell populations that were distinct from, but in addition to, sIgM^{int} populations observed after similar treatment of wild-type splenocytes. Loss of IL10 expression in dnRAG1 mice had no significant effect on B10-like B cell expansion or the frequency of PtC⁺ B cells. Compared to IL10^{+/+} dnRAG1 mice, levels of serum IgM, but not serum IgG, were highly elevated in some naïve IL10^{-/-} dnRAG1 mice, and was correlated with a significant increase in serum BAFF levels. Differentiation of sIgM^{int} B cells from LPS-stimulated dnRAG1 splenocytes was enhanced by loss of IL10 expression and IL10 blockade, but was suppressed by treatment with recombinant IL10. *In*

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Authorship contribution

V.L.P., A.N.W., R.L.S., G.A.P., and P.C.S. designed experiments, V.L.P., A.N.W., R.L.S., Q.Z.L., and M.Y. performed experiments and data analysis, V.L.P., A.W., and P.C.S. wrote the manuscript, and all authors reviewed, edited, and approved the manuscript.

Disclosure of conflicts of interest

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vitro LPS-induced differentiation and antibody production was inhibited by treatment with JAK/STAT inhibitors or a synthetic corticosteroid, independent of IL10 expression and genotype. Taken together, these data suggest that IL10 expression in dnRAG1 mice maintains suppression of IgM levels in part by inhibiting BAFF production, and that regulatory B10-like B cells, through the provision of IL10, constrains B cell differentiation in response to mitogenic stimuli. Furthermore, autoantibody profiling raises a possible link between CD5⁺ B cell expansion and autoantibodies associated with autoimmune complications observed in lupus and lupus-related disorders.

Keywords

IL10; CD5⁺ B cells; Regulatory B cells; B10 B cells; Autoantibody; Natural antibody; BAFF

1. Introduction

The provision of IL10 by regulatory subsets of B cells (Bregs) has become a well-established mechanism by which B cells assist in restraining excessive immune responses [1, 2]. However, IL10 is also an important cofactor for promoting B cell proliferation, differentiation, and antibody production [3–5]. Given the pleiotropic effects of IL10, it is perhaps not surprising that different autoimmune disorders are variously influenced by IL10 levels. For example, Bregs are known to be a critical source of IL10 that can restrain pathogenesis in autoimmune diseases such as inflammatory bowel disease [6], collagen-induced arthritis [7], and experimental autoimmune encephalomyelitis [8]. By contrast, some diseases characterized by autoantibody production, such as systemic lupus erythematosus (SLE), show a positive correlation between serum IL10 levels and disease severity [9, 10]. Interestingly, despite this correlation, global loss of IL10 expression in lupus-prone mice has been shown to accelerate disease progression [11]. This raises the question of whether IL10 normally functions to restrain or promote antibody production from autoreactive B cells.

Bregs can be identified based on their ability to express IL10 after stimulation by various factors that include toll-like receptor and CD40 agonists, and pro-inflammatory cytokines [2]. Bregs encompass many phenotypically diverse populations of B cells [2, 12]. Of particular relevance for this study is the description of a natural population of Bregs in mice, termed B10 B cells, which express IL10 in response to stimulation by LPS in the presence of phorbol ester and ionomycin, and predominantly exhibit a CD1d^{hi}CD5⁺ phenotype [13]. The frequency of B10 B cells under normal conditions is generally low, ranging from 1–3%, but is modestly expanded in mice predisposed to developing autoimmune disease, such as MRL-lpr and NZB/NZW mice [12]. B10 B cells have also been reported to increase in mice predisposed to accumulating populations of CD5⁺ B cells without an associated progression to autoimmunity [14]. The role of B cell-intrinsic IL10 expression in the latter scenario remains unclear, but since CD5⁺ B cells are often enriched in autoreactive B cell receptor specificities [15], the expansion of IL10-competent cells in these populations may represent a mechanism to help restrain autoantibody production in these animals. If so, disrupting IL10 expression in this context may unleash B cells to produce autoantibodies and promote the development of autoimmune disease.

We previously generated transgenic mice expressing catalytically inactive RAG1 (dnRAG1) that show an expanded CD5⁺ B cell subset, particularly prevalent in spleen and peritoneal cavity [16]. The splenic CD5⁺ B cells in dnRAG1 mice share features of splenic B1a and B10 B cells with respect to phenotype, gene expression profile, and IL10-competence [16–18]. The CD5⁺ B1a subset is thought to be the major source of natural antibody production [19]. Despite the accumulation of CD5⁺ B cells in dnRAG1 mice, these animals exhibit a profound immunoglobulin deficiency [16]. We previously showed that the CD5⁺ B cells in dnRAG1 mice display a restricted B cell receptor repertoire with biased J κ 1 usage consistent with a defect in B cell receptor editing [16]. As a result, we surmised these cells exhibit self-reactivity. In support of this possibility, we show here that a subset of splenic CD5⁺ B cells in dnRAG1 mice binds the autoantigen phosphatidylcholine (PtC). We further find that lipopolysaccharide (LPS)-stimulated dnRAG1 splenocytes produce higher levels of IgM than similarly treated wild-type (WT) splenocytes, and these secreted antibodies are enriched in reactivity toward autoantigens, including some associated with systemic lupus erythematosus (SLE), anti-phospholipid syndrome (APS), and autoimmune thyroiditis (AT). This outcome is correlated with the detection of distinctive sIgM^{hi} cells in LPS-stimulated splenocyte cultures from dnRAG1 mice.

Based on these results, we hypothesized that B10-like B cells expand as a mechanism to restrain potentially autoreactive B cells which cannot be salvaged by receptor editing and may not be subjected to clonal deletion. To test this possibility, we bred the dnRAG1 transgene onto an IL10-deficient strain background and showed that loss of IL10 expression in dnRAG1 mice does not affect CD5⁺ B cell accumulation, nor the frequency of PtC⁺ B cells in the spleen. Interestingly, however, loss of IL10 expression restored serum levels of IgM in some dnRAG1 mice, but had no effect on IgG levels. This outcome was correlated with a significant increase in levels of serum BAFF, which is known to positively regulate IgM production in response to thymus-independent antigens. Furthermore, we observed that expansion of c^{hi}sIgM^{int} plasma cells from WT and dnRAG1 splenocytes stimulated with LPS, but not their c^{hi}sIgM^{hi} counterparts detected in dnRAG1 cultures, was sensitive to IL10 levels. Finally, we provide evidence that LPS-induced B cell differentiation and antibody production is impaired by treatment with JAK1 and STAT3 inhibitors or a synthetic corticosteroid, but this effect is independent of IL10 receptor-dependent signaling. We discuss the implications of these results on our understanding of regulatory B cell function and the origin of autoantibodies associated with autoimmune complications in SLE and related disorders.

2. MATERIALS AND METHODS

2.1 Mice.

Transgenic dnRAG1 mice, described previously by our laboratory [16], were bred to *Il10^{tm1Cgn/J}* mice [20] (obtained from Jackson Laboratory; IL10^{-/-} mice henceforth); both strains are on a C57Bl/6 background. Non-transgenic (WT) IL10^{+/-} and dnRAG1 IL10^{+/-} offspring were crossed to generate cohorts of WT and dnRAG1 mice on *Il10^{+/+}* or *Il10^{-/-}* backgrounds for analysis. Mice were housed under specific pathogen-free conditions in microisolator cages in an AAALAC certified animal facility in accordance with university

and federal guidelines. All animal protocols were approved by the Creighton University Institutional Animal Care and Use Committee.

2.2 Antibodies

The following antibodies and clones were used: BD Biosciences (San Jose, CA) anti-B220-PE, or -CF594 (RA3–6B2), anti-CD19-APC-Cy7 (1D3), anti-CD23-biotin (B3B4), anti-CD43-biotin (S7), anti-NK1.1-PE-Cy7 (PK136), anti-CD4-APC-Cy7 (GK1.5); Biolegend (San Diego, CA) anti-CD21-APC-Cy7 (7E9), anti-CD138-PerCP-Cy5.5 (281–2); eBioscience (San Diego, CA) anti-CD19-A700 (1D3), anti-CD5-PE (53–7.3), anti-CD3-APC (145–2C11), anti-CD8-A700 (53–6.7), anti-CD4-A700 (GK1.5), anti-CD49b-PE-Cy7 (DX5), anti-CD93-PE-Cy7 (AA4.1), anti-IgM-FITC (II/41), anti-IgM-APC (II/41), anti-IgD-FITC (11–26c). Samples stained with biotinylated antibodies were detected using streptavidin-BV510 or -BUV737 (BD Biosciences).

2.3 Flow cytometry

Bone marrow cell suspensions were made by flushing two femurs and tibias with RF10-M (RPMI 1640 [Invitrogen] containing 2 mM l-glutamine, 10% fetal calf serum [Invitrogen], 1,000 U-μg/mL penicillin–streptomycin [Fisher Scientific, Waltham, MA], 14 mM 2-mercaptoethanol, 20 mM HEPES [GIBCO/Invitrogen, Carlsbad, CA]) using a 25-gauge needle. Single-cell suspensions from spleen were made by mincing the tissue with two needles in RF10-M and passing the material through a needleless syringe. Red blood cells were lysed in TBAC buffer (140 mM ammonium chloride, 17 mM Tris-base, pH 7.2) on ice for five minutes, and quenched with an equal volume of RF10-M. Cells were centrifuged and resuspended in 1 mL of RF10-M and counted on a Model Z1 particle counter (Beckman Coulter, Fullerton, CA).

For analysis for cell surface antigens, samples containing 1×10^6 cells were treated with Fc-block reagent (anti-CD16/CD32, clone 93; eBioscience), and then incubated with antibody cocktails for thirty minutes on ice in the dark. Cells were then washed with PBS4 (Dulbecco's phosphate buffered saline, 4% heat inactivated FBS), incubated with the desired streptavidin conjugate, washed again with PBS4, and finally fixed in 1% neutral buffered formalin in PBS.

To analyze intracellular antigens, cells were first treated with Fc block reagent and stained with antibodies to detect surface antigens as described above. For intracellular μ H chain detection, a second round of staining with 10-fold excess anti-IgM-FITC (II/41) was performed to saturate remaining surface IgM. Next, cells were fixed and permeabilized using the Cytotfix/CytoPerm kit (BD Biosciences). After washing, cells were stained with eBioscience anti-IgM-APC (II/41), anti-IL10-APC (JES5–16E3), or control antibodies conjugated to APC (eBR2a or eB149/10H5; eBioscience). As controls, some samples were fixed but not permeabilized before staining.

PtC binding was detected by including 200nm Fluorescein-DHPE/DOPC/CHOL liposomes (1:400 dilution of 0.59 mg/mL stock; FormuMax Scientific) in the antibody cocktail. As controls, some samples were stained with antibody cocktails containing fluorescein-PtC liposomes and a 10-fold excess of non-fluorescent PtC liposomes as a competitor.

A total of 100,000 events were acquired for each sample using a BD FACS Aria flow cytometer (BD Biosciences) or a ZE5 Cell Analyzer (BioRAD). FloJo software was used for data analysis (TreeStar, Ashland, OR).

2.4 Cell culture

Single-cell suspensions from spleen were adjusted to 4.0×10^6 cells/mL in sterile RF10M and 2.0×10^5 cells dispensed into wells of a sterile 96-well flat bottom plate (Corning #353072). The cells were mixed with an equal volume of RF10M lacking or containing the following reagents (source and final concentration shown in parentheses): LPS (Sigma, L2880, 10 μ g/mL), IL10 blocking antibody (R&D Systems, MAB417, 10 ng/mL), Rat IgG1 control for IL10 blocking antibody (R&D Systems, MAB005, 10 ng/mL in PBS), recombinant mouse IL10 (Biolegend, 575802, 100 ng/mL), ruxolitinib (Selleckchem, S1378, 10 nM), SH-4-54 (Selleckchem, S7337, 10 nM), or triamcinolone acetonide (Alfa Aesar, J6354803, 10 nM). DMSO was used as a vehicle control for the small molecule inhibitors. Cells were cultured for 72h at 37 °C in a humidified incubator with 5% CO₂.

2.5 Autoantibody profiling

Autoantibody reactivity against a panel of 124 autoantigens was measured using a microarray platform developed by the University of Texas Southwestern Medical Center (<https://microarray.swmed.edu/products/category/protein-array/>). The autoantigen array bearing 124 autoantigens and 4 control proteins was printed in duplicates onto nitrocellulose film slides (Grace Bio-Labs). WT and dnRAG1 splenocytes (both IL10^{+/+}; n=3/genotype) were cultured in the presence of LPS as described in section 2.4, and antibody concentrations in the resulting culture supernatants were normalized to 10 μ g/ml in PBST buffer. Samples were incubated with the autoantigen arrays, and autoantibodies were detected with cy3-labeled anti-mouse IgG and cy5-labeled anti-mouse IgM using a Genepix 4200A scanner (Molecular Devices) with laser wavelength of 532 nm and 635 nm. The resulting images were analyzed using Genepix Pro 6.0 software (Molecular Devices). The median of the signal intensity for each spot was calculated, the local background around the spot was subtracted, and the values obtained from duplicate spots were averaged. The background-subtracted signal intensity of each antigen was normalized to the average intensity of the total mouse IgG or IgM, which was included on the array as an internal control. Finally, the net fluorescence intensity (NFI) for each antigen was calculated by subtracting a PBS control which was included for each experiment as negative control. The signal-to-noise ratio (SNR) was used as a quantitative measurement of the true signal above background noise. SNR values equal to or greater than 3 were considered significantly higher than background, and therefore true signals. In comparing values obtained from WT or dnRAG1 culture supernatants, only those autoantigens for which all three samples from both genotypes gave an NFI > 0 and SNR > 3 were included in the analysis. For each autoantigen included in the analysis, the average of the three values for each genotype and the fold-difference between dnRAG1 and WT samples were calculated.

2.6 ELISA

Commercial sandwich ELISA kits were used according to manufacturer's instructions to measure IgM and IgG concentrations in serum and culture supernatants (Zeptometrix) and to measure serum BAFF levels (R&D Systems).

For PtC-specific ELISAs, 96-well MaxiSorp plates (Nunc) were coated with 1,2-Dioleoyl-sn-glycero-3-phosphocholine (100 mg/L, Sigma-Aldrich) in a 4:1 solution of methanol and chloroform and left to evaporate for eight hours at 4 °C [21]. For all other autoantigen-specific ELISAs, plates were coated for eight hours with the following autoantigens in 50 mM carbonate buffer (0.159% Na₂CO₃ and 0.293% NaHCO₃, pH 9.6): thyroid peroxidase (TPO; 0.25 mg/L, Surmodics), Smith D2 (0.5 mg/L, Surmodics), thyroglobulin (0.5 mg/L, Surmodics), proliferating cell nuclear antigen (PCNA; 0.5 mg/L, Surmodics), beta-2 glycoprotein I (β2-GPI; 0.5 mg/L, Surmodics) [22–24]. After coating, plates were washed twice with phosphate buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, pH 7.3), then blocked overnight at 25 °C with 1% bovine serum albumin (BSA, BioWorld) in PBS with 0.5% Tween-20 (PBS-T). Plates were washed three times with PBS-T before adding cell culture supernatants diluted 1:10 in PBS-T with 0.1% BSA for two hours at 25 °C. Plates were washed three times with PBS-T. Horseradish peroxidase conjugated goat anti-mouse IgM or IgG (Invitrogen), diluted 1:1000 in PBS-T and pre-adsorbed with 10% RF10-M medium for 30 minutes, was added to the plates and incubated for two hours at 25 °C. Plates were washed a final three times in PBS-T and then 1-Step™ Ultra TMB-ELISA Substrate Solution (50 µL, ThermoFisher) was added for 30 minutes at 25 °C in the dark [25]. The reaction was quenched by adding 2M sulfuric acid (50 µL), and the absorbance was measured at 450 nm on a Synergy HTX plate reader using Gen5 Imager Software (BioTek, Software version 2.08.13). Data was analyzed using Prism Software (GraphPad, v4.03).

2.7 Statistics

Data are presented as mean values ± standard error of the means. Collected data were subjected to analysis of variance and post hoc testing using the PASW Statistics 24.0 software package (SPSS Inc., Chicago, IL). Unless otherwise indicated, differences with a *p*-value of 0.05 by Bonferroni post-hoc testing are considered statistically significant.

3. Result

3.1 IL10 expression is required to support normal levels of B cell maturation and maintain suppressed serum IgM levels in dnRAG1 mice, but is dispensable for CD5⁺ B cell accumulation in these animals

We previously reported that dnRAG1 mice show a marked expansion of a splenic B1-like CD5⁺ B cell population that displays a biased immunoglobulin gene repertoire and is highly enriched with IL10-competent cells [16, 18]. These animals also exhibit a striking deficit in basal serum IgM and IgG levels [16, 18]. To specifically determine whether IL10 expression is required to sustain CD5⁺ B cell expansion and hypogammaglobulinemia in dnRAG1 mice, we bred the dnRAG1 transgene onto an IL10-deficient strain background to produce WT and dnRAG1 that were proficient or deficient in IL10 production (called IL10^{+/+} WT,

IL10^{-/-} WT, IL10^{+/+} dnRAG1, and IL10^{-/-} dnRAG1 mice, respectively). These animals were compared at 8–10 weeks of age for the frequency of developing and mature B cell subsets in the bone marrow and spleen using flow cytometry, and levels of serum IgM and IgG as measured by ELISA.

In the bone marrow, we found that IL10-deficient WT and dnRAG1 mice had ~20% fewer total lymphocytes, and only about half the number of B cells at or beyond the pre-B cell (B220⁺CD43⁻) stage of development, as compared to their IL10-proficient counterparts (Table S1). In the spleen, by contrast, we detected no significant differences in subsets of transitional (T1–T3), mature (marginal zone and follicular), and CD19⁺CD5⁺ B cells (Fig. 1A, and Table S1). Hence, IL10 expression is necessary to support normal levels of B cell maturation, but is not responsible for supporting the expansion of splenic CD5⁺ B cells in dnRAG1 mice.

When we compared serum IgM and IgG levels by ELISA, IL10^{+/+} dnRAG1 mice showed significantly reduced levels of serum IgM and IgG compared to IL10^{+/+} WT mice, as expected from our previous studies [16](Fig. 1B). Interestingly, however, whereas loss of IL10 expression had no significant effect on serum IgM and IgG levels in the WT background, in the dnRAG1 background, 9/14 (64%) animals showed serum IgM levels that were greater than the highest level observed in IL10^{+/+} dnRAG1 mice. While the large variation among IL10^{-/-} dnRAG1 animals did not make the difference between IL10^{+/+} dnRAG1 mice statistically significant, there was no longer any significant difference between IL10^{-/-} dnRAG1 mice and IL10^{+/+} WT or IL10^{-/-} WT mice either (Fig. 1B). By contrast, the distribution of IgG levels was not different between IL10^{+/+} dnRAG1 and IL10^{-/-} dnRAG1 mice, and the latter remained significantly lower than both IL10^{+/+} WT and IL10^{-/-} WT mice (Fig. 1B). Thus, these data suggest that IL10-deficiency can restore serum levels of IgM, but not IgG, in some naïve dnRAG1 mice.

3.2 Loss of IL10 expression significantly increases BAFF levels in dnRAG1 mice.

The apparent selective restoration of serum IgM levels to basal levels in the majority of IL10^{-/-} dnRAG1 mice led us to speculate that loss of IL10 expression in dnRAG1 mice released a constraint on IgM production in these animals. Based on evidence suggesting BAFF plays a key role in supporting T cell-independent IgM responses [26–28], we considered the possibility that BAFF levels may be suppressed in IL10^{+/+} dnRAG1 mice, and loss of IL10 expression may lead to upregulation of BAFF in this background. Consistent with this possibility, we found that serum BAFF concentrations were slightly lower in dnRAG1 IL10^{+/+} mice compared to WT IL10^{+/+} mice, and loss of IL10 expression in dnRAG1 mice led to a significant increase in BAFF levels as compared to dnRAG1 IL10^{+/+} mice (Fig 1C). Loss of IL10 expression in the WT background also had a similar effect. Thus, IL10-deficiency in dnRAG1 mice significantly elevates basal serum BAFF levels.

3.3 Lipopolysaccharide induces a robust IgM response in dnRAG1 splenocytes associated with differentiation into plasma cell subsets distinguishable by surface IgM phenotype,

The hypogammaglobulinemia evident in IL10^{+/+} dnRAG1 mice raised the possibility that B cells from IL10^{+/+} dnRAG1 mice are intrinsically less responsive to stimulation and antibody production than WT mice and IL10^{-/-} dnRAG1 mice. To test this possibility, splenocytes from IL10^{+/+} or IL10^{-/-} mice on a WT or dnRAG1 mice background were compared to determine levels of IgM and IgG produced in response to LPS stimulation (Fig. 2A). Spontaneous secretion of IgM, while low for all genotypes, was slightly higher in IL10-deficient genotypes, but the differences were not statistically significant. Interestingly, LPS-stimulated splenocytes from IL10^{+/+} dnRAG1 mice produced significantly more IgM but significantly less IgG than splenocytes from WT mice on either the IL10^{+/+} or IL10^{-/-} backgrounds. By contrast, loss of IL10 expression had no effect on levels of LPS-induced IgM and IgG produced in the WT background, but on the dnRAG1 background, IL10-deficient splenocytes produced less IgM and more IgG than their IL10-proficient counterparts such that the differences compared to WT mice on the IL10^{+/+} and IL10^{-/-} backgrounds were no longer statistically significant.

To determine whether the high levels of IgM produced by LPS-treated IL10^{+/+} dnRAG1 splenocytes relative to IL10^{+/+} WT splenocytes was associated with an increased frequency of plasma cells, the cultured cells were analyzed after LPS treatment by flow cytometry to characterize the responding B cells for evidence of cells with a plasma cell phenotype (IgM⁺cp^{hi}CD138⁺) [29] (Fig. 2B). LPS-stimulated splenocytes from IL10^{+/+} WT mice showed a major population of phenotypically IgM^{int}cp^{int} cells, and a smaller population of IgM^{int}cp^{hi} cells that are nearly absent in untreated cultures (Fig. 2B). The cp staining pattern is antigen-specific, as it is not detected using an isotype control antibody, and requires permeabilization, because staining is not apparent when cells were only subjected to fixation (Fig. S1A). The latter population exhibits upregulated CD138 expression, consistent with a plasma cell designation (Fig. S1B). By contrast, LPS-stimulated splenocytes from IL10^{+/+} dnRAG1 mice showed the same two IgM^{int} populations as observed in WT mice, as well as two additional populations with a similar pattern of cp expression but with a distinctive IgM^{hi} phenotype. Interestingly, loss of IL10 expression in the dnRAG1 background led to a proportional increase in the frequency of IgM^{int}cp^{hi} B cells relative to IgM^{hi}cp^{hi} B cells, but had little effect on the IgM^{int}cp^{hi} B cells population on the WT background (Fig. 2B). Taken together, these data demonstrate a correlation between the robust LPS-induced IgM response in dnRAG1 splenocytes and the presence of IgM^{hi} plasma cells in the cell culture, and indicate that the presence of IL10 restrains LPS-induced differentiation of IgM^{int}cp^{hi} B cells and production of IgG.

3.3 LPS-induced plasma cell subsets from dnRAG1 mice are differentially responsive to IL10 levels, inhibitors of IL10 receptor-dependent signaling, and corticosteroid treatment.

To further confirm that LPS-induced formation of IgM^{int}cp^{hi} B cells are differentially responsive to IL10 levels compared to IgM^{hi}cp^{hi} B cells, we treated the cell cultures with either recombinant IL10 or, alternatively, with IL10-blocking antibody (Fig. 3A). Consistent with the finding that IL10^{-/-} dnRAG1 splenocytes support more LPS-induced IgM^{int}cp^{hi} B

cell differentiation than IL10^{+/+} dnRAG1 splenocytes, supplementing IL10^{+/+} dnRAG1 splenocyte cultures with recombinant IL10 during LPS activation reduced the proportion of IgM^{int}cμ^{hi} B cells, whereas the converse was true when cultures were supplemented with IL10-blocking antibody. By contrast, neither treatment had significant effects on the relative proportion of the IgM^{hi}cμ^{hi} B subset. Notably, isotype antibody control treatments resembled their counterparts stimulated with LPS only. These data suggest that direct, IL10-dependent signaling negatively regulates IgM^{int}cμ^{hi} B cell differentiation.

Since IL10-blocking antibody was found to promote LPS-induced IgM^{int}cμ^{hi} B cell differentiation in dnRAG1 splenocytes, we next tested whether inhibitors of IL10-dependent signaling might have a similar effect. Because responses to IL10 receptor stimulation are thought to be mediated primarily via JAK1/STAT3 activation [30], we treated LPS-stimulated splenocyte cultures from WT and dnRAG1 mice with small molecule inhibitors designed to specifically target JAK1 (Ruxolitinib/INCB018424) [31] or STAT3 (SH-4-54) [32]. We also tested triamcinolone acetonide (TA), a synthetic corticosteroid known to inhibit B cell responses to mitogenic stimulation [33, 34]. Interestingly, in both IL10^{+/+} WT and IL10^{+/+} dnRAG1 splenocyte cultures, SH-4-54 treatment was found to block the early response to LPS, as the phenotypic profile of the treated B cells resembles untreated cells (Fig. 3A). In contrast, both LPS-treated cultures supplemented with Ruxolitinib showed the presence of B cells that were phenotypically distinct from those in untreated cultures, but with cμ staining more broadly distributed among the IgM^{int} population than in the LPS +DMSO control samples. Finally, compared to Ruxolitinib, treatment of LPS-stimulated dnRAG1 splenocyte cultures with TA more selectively impaired the differentiation of the sIgM^{hi} B cell subsets, whereas the effects on the sIgM^{int} populations were more modest.

To establish how the various treatments affect antibody production, we also measured IgM and IgG levels in the culture supernatants (Fig. 3B). Addition of recombinant IL10 substantially diminished IgM levels produced by both LPS-stimulated IL10^{+/+} WT and IL10^{+/+} dnRAG1 splenocytes, whereas its effect on IgG production was not significant. By contrast, blocking IL10 antibody had opposing effects on IgM and IgG production in LPS-stimulated IL10^{+/+} dnRAG1 splenocytes compared to isotype control-treated samples, whereas IgM and IgG levels remained unchanged in similarly treated samples from WT mice. With respect to the effect of small molecule inhibitors, SH-4-54 treatment effectively blocked LPS-induced antibody production from both IL10^{+/+} WT and IL10^{+/+} dnRAG1 splenocytes, which is consistent with its substantial inhibition of B cell differentiation in both cases. Similar effects were observed for samples treated with TA, and were slightly milder for samples treated with ruxolitinib.

3.5 A subset of splenic CD5⁺ B cells in dnRAG1 mice recognizes the autoantigen PtC

Our previous studies suggested that dnRAG1 mice exhibit a defect in receptor editing, which in principle should enforce B cell autoreactivity. Since the CD5⁺ B cells accumulating in dnRAG1 exhibit phenotypic similarities to B1a and B10 B cells, and a subset of peritoneal cavity (PerC) B1a are known to recognize the autoantigen PtC [35], we stained IL10^{+/+} WT and IL10^{+/+} dnRAG1 splenocytes with FITC-labeled PtC-containing liposomes to determine whether the splenic CD5⁺ B1a-like B cell population in dnRAG1 mice contains cells that

recognize this autoantigen (Fig. 4). Whereas IL10^{+/+} WT mice show very few PtC⁺ B cells in the spleen, IL10^{+/+} dnRAG1 mice harbor ~3% PtC⁺ B cells in the spleen. Of the splenic PtC⁺ B cells detected in IL10^{+/+} dnRAG1 mice, >90% are also CD5⁺. This population was not evident when splenocytes were stained with unlabeled PtC liposomes (data not shown). Furthermore, the mean channel fluorescence of PtC staining was significantly diminished (~5-fold) when the antibody cocktail containing the Fluorescein-labeled PtC liposomes was spiked with a 10-fold excess of unlabeled PtC liposomes, supporting the conclusion that the PtC binding observed in this assay is antigen-specific. However, like CD5⁺ B cells in dnRAG1 mice in general, the relative frequency of PtC⁺ B cells in the spleen was not significantly affected by loss of IL10 expression. In the PerC, the absolute number of PtC⁺ B cells was slightly higher in IL10^{+/+} dnRAG1 mice than their IL10^{+/+} WT counterparts, but the percentage of CD5⁺ B cells that stained PtC⁺ was actually modestly increased in the IL10-deficient background independent of dnRAG1 expression (Fig. 4).

3.6 Autoantigen array screening reveals selectivity of LPS-induced IgM and IgG antibodies from dnRAG1 mice toward certain autoantigens.

Since PtC binding by a subset of CD5⁺ B cells from IL10^{+/+} dnRAG1 mice established their potential reactivity toward autoantigen, we next sought an unbiased method to evaluate the profile of autoantigens recognized by antibodies produced from IL10^{+/+} dnRAG1 mice. Toward this end, culture supernatants recovered after LPS stimulation of IL10^{+/+} WT and IL10^{+/+} dnRAG1 splenocytes (n=3/genotype) were screened for IgM and IgG autoantibody reactivity toward a panel of 124 autoantigens using an array-based approach. The fold-difference in signal between IL10^{+/+} dnRAG1 and IL10^{+/+} WT samples was determined for each autoantigen, and the values were then ranked from highest to lowest, with the 20 autoantigens exhibiting the greatest fold difference in reactivity for IgM and IgG shown in Fig. 5A (see Table S2 for full list). Among these autoantigens, half are found on both lists (numbers in parentheses show ranking for IgM and IgG, respectively), including Smd2 (1/11), thyroid peroxidase (TPO) (2/7), vitronectin (3/8), liver cytosol type 1 (4/3), matrigel (7/10), complement C3b (11/18), aquaporin 4 (AQP4) (13/17), β 2 glycoprotein 1 (15/6), proliferating cell nuclear antigen (PCNA) (16/15), and complement C9 (17/2). Differences between IL10^{+/+} WT and IL10^{+/+} dnRAG1 reactivity were found to be statistically significant for some, but not all, of the autoantigens listed (Fig. 5B). Smd2, PCNA, and complement C3b and C9 are targeted autoantigens in systemic lupus erythematosus (SLE), whereas TPO and β 2 glycoprotein 1 are targets in autoimmune thyroiditis (AT) and anti-phospholipid syndrome (APS), respectively, but are not restricted to these disorders. Indeed, SLE patients may have autoantibodies that overlap with those associated with other autoimmune disorders as well, including some listed here [36–39]. To follow up the autoantigen array screening results, we used antigen-specific ELISAs to confirm the reactivity of IL10^{+/+} dnRAG1 antibodies produced after *in vitro* LPS stimulation toward several of these autoantigens (Fig. 5C). Additional ELISAs were used to test reactivity toward PtC, which was not evaluated in the array screening assay, and thyroglobulin, which is another target of autoantibodies in AT and was present among the top 20 autoantigens that are enriched in IgG autoreactivity in LPS-stimulated dnRAG1 splenocytes. Taken together, these data associate CD5⁺ B cell accumulation in IL10^{+/+} dnRAG1 mice with autoreactivity toward a spectrum of autoantigens targeted in a variety of autoimmune disorders.

4. Discussion

In previous studies [16–18], we have shown that IL10^{+/+} dnRAG1 mice accumulate a population of splenic CD5⁺ B cells which resembles splenic B1 cells with respect to phenotype and gene expression profile, and contains a high frequency of IL10-competent B cells (50–60% of CD5⁺ B cells). The CD5⁺ B cell repertoire is enriched in germline-encoded heavy and light chain genes, the latter of which is biased toward J κ 1 gene segment usage, and is selected based on antigenic specificity, as enforced expression of a site-directed anti-dsDNA heavy chain transgene in dnRAG1 mice blocks the development of this population [16]. Furthermore, we show here that a subset of the splenic CD5⁺ B cells in dnRAG1 mice recognize PtC, an autoantigenic specificity enriched in CD5⁺ peritoneal B1 cells [35], and IgM and IgG antibodies produced by IL10^{+/+} dnRAG1 splenocytes after LPS stimulation *in vitro* react toward a spectrum of autoantigens, including those targeted in autoimmune disorders such as SLE, AT, and APS. Based on these data, we propose a model in which dnRAG1 expression interferes with the efficiency of secondary V(D)J rearrangements that edit B cell receptor (BCR) specificity in response to self-reactivity. As a result, bone marrow-derived B cell progenitors that would normally be subjected to receptor editing to remove autoantigenic specificities which could otherwise be positively selected in the peritoneal B1 lineage may be prevented from doing so. If such receptor specificities cannot trigger clonal deletion upon BCR engagement of autoantigen, the cells may be shunted into a pathway that causes them to adopt a CD5⁺ splenic B1a phenotype and become IL10 competent as one mechanism to suppress potential autoantibody production. The observation that IL10^{+/+} dnRAG1 mice, despite the expansion of splenic B1a-like cells, show a striking reduction in natural antibody levels compared to wild-type mice is consistent with this idea.

If IL10 is required to direct or sustain splenic B1-like cell expansion in IL10^{+/+} dnRAG1 mice and/or maintain suppression of natural antibody production in these animals, we might expect loss of IL10 expression in dnRAG1 mice to reduce the frequency of the splenic B1-like B cells and/or restore natural antibody levels. However, a comparison of IL10^{+/+} dnRAG1 mice and IL10^{-/-} dnRAG1 mice revealed no significant differences in the absolute number of the splenic B1-like B cells between the two strains. Thus, reminiscent of early studies of IL10^{-/-} mice showing that the frequency of peritoneal B1 cells is largely unaffected by loss of IL10 expression [20], IL10 is also dispensable for the expansion of splenic B1 cells in dnRAG1 mice. Interestingly, however, IL10^{-/-} dnRAG1 mice did show increased serum IgM levels in a majority of mice compared to IL10^{+/+} dnRAG1 mice, suggesting a role for IL10 for suppressing IgM production in the dnRAG1 background. This was not the case for serum IgG. Previous studies showed that loss of IL10 expression had little effect on basal levels of serum immunoglobulins in a WT strain background [20], which we also observe here. Thus, the effect of IL10 loss on serum immunoglobulins in dnRAG1 mice must be attributed to the distinctive predominance of splenic B1-like B cells in these animals, and suggests that the B cells in IL10^{+/+} dnRAG1 are proficient in producing IgM, but being actively suppressed from doing so in an IL10-dependent manner. Our finding that levels of BAFF, a key regulator of T cell-independent IgM responses [26–28], is diminished in IL10^{+/+} dnRAG1 mice, but elevated in IL10^{-/-} WT and IL10^{-/-}

dnRAG1 mice, may provide an important clue to explain how suppression of IgM production is sustained in IL10^{+/+} dnRAG1 mice. In this model, the provision of IL10 suppresses the production of BAFF, which in turn, constrains IgM secretion. However, the precise mechanism by which IL10 mediates this effect remains to be elucidated.

Our detection of a PtC⁺ subset within the splenic and PerC CD5⁺ B cell population in IL10^{+/+} dnRAG1 mice establishes that these cells possess reactivity toward autoantigen. This is further supported by analysis of LPS-induced IgM and IgG antibodies from IL10^{+/+} dnRAG1 mice, showing they exhibit reactivity toward PtC, as well as a spectrum of other autoantigens associated with various autoimmune disorders, including SLE, AT, and APS. In this regard, it is noteworthy that expansion of CD5⁺ B cells is frequently observed in certain autoimmune diseases, including SLE, Sjögren's syndrome, and rheumatoid arthritis [40–42]. Patients afflicted with SLE and other lupus-related disorders, show increased susceptibility to certain autoimmune complications, such as hypothyroidism [36, 37] and cytopenias [43]. The underlying genetics that predispose individuals to these disorders and their complications is complex and not fully understood, and disease manifestations may be influenced by various environmental triggers [44]. Of particular relevance to this study, infections have been implicated as triggers for autoimmune manifestations in lupus-prone individuals [44, 45], and may also underlie complicating autoimmune phenomena [46, 47]. While some reports [48] suggest that high affinity autoantibodies in SLE (specifically reactive to ssDNA) are not produced by CD5⁺ B1-like B cells, but rather by CD5[−] B cells presumed to be B2 in origin, this evidence does not exclude the possibility that distinct autoantigen-reactive B cells contribute to other primary or complicating autoimmune manifestations in this disease. Indeed, the autoantigen reactivity profile of LPS-elicited IgM and IgG antibodies from IL10^{+/+} dnRAG1 splenocytes includes thyroid and membrane autoantigens, and leads us to speculate that some autoimmune complications of SLE and related disorders, particularly hypothyroidism and cytopenias, could be attributed to infection-induced stimulation and autoantibody production by CD5⁺ B cells that tend to expand in these diseases. The possibility that IL10^{+/+} dnRAG1 mice may be susceptible to developing autoimmune phenomena in response to mitogenic stimulation will be a topic of future study.

If this hypothesis is true, our results suggest that CD5⁺ B cells may represent a druggable target to suppress a subset of autoimmune manifestations in lupus-related disorders. Toward this end, our finding that TA shows some selectivity in blocking LPS-induced differentiation of sIgM^{hi} B cells in splenocytes from IL10^{+/+} dnRAG1 mice may provide a partial explanation (and justification) for its efficacy in treating autoimmune disorders where CD5⁺ B cells could be implicated in some disease complications. By contrast, JAK1 and STAT3 inhibitors, though effective at blocking LPS-induced differentiation *in vitro* (albeit at different steps), showed little selectivity in blocking *in vitro* LPS-induced B cell differentiation of splenocytes from IL10^{+/+} WT and of IL10^{+/+} dnRAG1 mice. In this model, these drugs also showed no dependence on IL10 expression, suggesting they inhibit a pathway that does not activate IL10 receptor signaling.

Finally, the observation that supplementing LPS-stimulated splenocyte cultures from IL10^{+/+} WT and IL10^{+/+} dnRAG1 mice with recombinant IL10 suppresses plasma cell differentiation of sIgM^{int} B cells *in vitro*, and supplementing with blocking IL10 antibody

had the opposite effect, is consistent with a previous study demonstrating that IL10 treatment suppresses LPS-induced B cell proliferation *in vitro* [49]. The comparatively small effect that either of these treatments had on sIgM^{hi} B cell differentiation in IL10^{+/-} dnRAG1 cultures suggests this population is intrinsically more resistant to the regulatory effects of IL10. This idea is plausible because the CD5⁺ B population in dnRAG1 mice is enriched in IL10 competent cells which may not react to the IL10 they produce [18]. This observation may provide insight into how regulatory B cells which produce IL10 in response to various stimuli (including LPS) could contribute toward negatively regulating antibody production by other B cells in response to thymus-independent antigens.

4.1 Conclusions.

In summary, we have provided evidence that IL10 is required to suppress basal IgM production and restrain serum BAFF levels in dnRAG1 mice, but is dispensable for CD5⁺ B cell accumulation and PtC reactivity in these animals. We also show that *in vitro* LPS stimulation of splenocytes from dnRAG1 mice induces the differentiation of a distinctive population of sIgM^{hi} B cells, in addition to the sIgM^{int} B cells observed in similarly stimulated WT splenocytes. This LPS-induced differentiation of sIgM^{hi} B cells in dnRAG1 splenocytes is associated with the production of antibodies toward a variety of autoantigens, including some identified as targets of autoantibodies in SLE, AT, and APS. Expansion of plasma cells from the sIgM^{int} subset is more sensitive than those from the sIgM^{hi} subset to manipulation of IL10 levels. Antibody production by LPS-stimulated splenocytes is significantly reduced by treatment with the synthetic corticosteroid TA and inhibitors targeting the JAK1-STAT3 signaling pathway, with TA exhibiting the most selective reduction on sIgM^{hi} cells. Taken together, these results suggest that regulatory B cells, through the provision of IL10, assist in restraining self-reactive B cells from producing autoantibodies. These results also implicate CD5⁺ B cells as a possible source for autoantibodies that may trigger autoimmune complications in lupus-related disorders, and suggest a therapeutic target for more selective treatments of these conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Transgenic mice expressing inactive RAG1 (dnRAG1 mice) develop autoreactive B cells
- *In vitro* differentiation of B cell subsets in dnRAG1 mice are distinctly regulated by IL10
- Loss of IL10 expression in dnRAG1 mice elevates IgM production and BAFF levels
- IL10 is dispensable for CD5⁺ B cell expansion and phosphatidylcholine binding in dnRAG1 mice
- JAK1/STAT3 inhibitors and corticosteroids block dnRAG1 B cell differentiation *in vitro*

Fig. 1. IL10 expression is dispensable for CD5⁺ B cell accumulation, but regulates serum IgM levels in dnRAG1 mice.

(A) Splenocytes from mice with the indicated genotype were analyzed by flow cytometry for CD5 and CD19 expression among gated lymphocytes (left panel). The percentage of cells in each gate is shown for representative animals. Summary data are presented in bar graph format and represented as mean ± SEM. Statistically significant differences are shown. (B) Serum IgM and IgG levels in mice with the indicated genotypes were measured by ELISA. (C) Serum BAFF levels in mice with the indicated genotypes were measured by ELISA. Statistically significant differences are shown.

Fig. 2. dnRAG1 splenocytes stimulated *in vitro* with LPS show robust IgM production associated with B cell differentiation to a sIgM^{hi} plasma cell subset.

(A) Splenocytes from mice with the indicated genotypes were cultured in the absence or presence of LPS (10 μ g/mL) for 72h. IgM and IgG concentrations in the culture supernatants were measured by ELISA. Statistically significant differences are shown. (B) Splenocytes cultured as in panel A were analyzed for surface IgM and cytoplasmic μ expression by flow cytometry as in Fig. 2. The percentage of cells in each gate is shown for representative animals. Summary data are presented in bar graph format and represented as mean $\pm$ SEM. Statistically significant differences are shown.

Fig. 3. LPS-induced plasma cell subsets and antibody production by WT and dnRAG1 splenocytes are differentially responsive to IL10 levels, inhibitors of IL10-dependent signaling, and corticosteroid treatment.

(A). Splenocytes from WT or dnRAG1 mice were cultured for 72h with or without LPS (10 μ g/mL) in the absence or presence of the following: recombinant IL10 (rIL10), IL10 blocking antibody (α -IL10 Ab) or isotype control (Iso Ab), or ruxolitinib (Rux), SH-4-54, triamcinolone acetonide (TA) or vehicle (DMSO), and plasma cell subsets were analyzed by flow cytometry as in Fig. 2B. Summary data for n=3 animals/genotype are presented in bar graph format and represented as mean $\pm$ SEM. Statistically significant differences using

the Tukey correction are indicated (*a*, vs LPS; *b*, vs LPS+Iso Ab; *c*, vs LPS+DMSO). (B) Concentrations of IgM and IgG in supernatants from splenocyte cultures shown in panel A were measured by ELISA.

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Fig. 4. A subset of splenic CD5⁺ B cells in dnRAG1 mice binds liposomes containing PtC. Splenocytes from the indicated mouse genotypes were stained with PtC-FITC liposomes in the absence or presence of 10-fold excess unlabeled PtC liposomes and the frequency of CD5⁺PtC⁺ cells among gated CD19⁺ lymphocytes was analyzed by flow cytometry. The percentage of cells in the CD5⁺PtC⁺ gate is shown for representative animals. Summary data are presented in bar graph format and represented as mean $\pm$ SEM. Statistically significant differences are shown.

Fig. 5. LPS-induced IgM and IgG antibodies from dnRAG1 splenocytes exhibit autoreactivity. (A) Supernatants from WT and dnRAG1 splenocytes cultured with LPS (10 μ g/mL) for 72h were screened for IgM and IgG reactivity against 124 autoantigens using an array-based approach as described in Materials and Methods. The fold-difference between the averaged signal values obtained from dnRAG1 and WT samples were ranked from highest to lowest. The 20 autoantigens showing the largest fold-difference are listed; those appearing on both lists are shaded. (B) Summary data for the 10 autoantigens found on both lists are presented in bar graph format and represented as mean signal value $\pm$ SEM. Statistically significant

differences in values ($p < 0.05$) between WT and dnRAG1 samples are indicated by an asterisk. (C) Supernatants from unstimulated and LPS-stimulated WT and dnRAG1 splenocytes were analyzed for IgM and IgG reactivity toward selected autoantigens by ELISA. Statistically significant differences between LPS-stimulated WT and dnRAG1 samples are indicated by an asterisk.

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