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In Vivo Detection of Peripherin-Specific Autoreactive B Cells during Type 1 Diabetes Pathogenesis

Nahir Garabatos,* Raimon Alvarez,* Jorge Carrillo,† Jorge Carrascal,† Cristina Izquierdo,* Harold D. Chapman,‡ Maximiliano Presa,* Conchi Mora,† David V. Serreze,‡ Joan Verdaguer,† and Thomas Stratmann*

Autoreactive B cells are essential for the pathogenesis of type 1 diabetes. The genesis and dynamics of autoreactive B cells remain unknown. In this study, we analyzed the immune response in the NOD mouse model to the neuronal protein peripherin (PRPH), a target Ag of islet-infiltrating B cells. PRPH autoreactive B cells recognized a single linear epitope of this protein, in contrast to the multiple epitope recognition commonly observed during autoreactive B cell responses. Autoantibodies to this epitope were also detected in the disease-resistant NOR and C57BL/6 strains. To specifically detect the accumulation of these B cells, we developed a novel approach, octameric peptide display, to follow the dynamics and localization of anti-PRPH B cells during disease progression. Before extended insulitis was established, anti-PRPH B cells preferentially accumulated in the peritoneum. Anti-PRPH B cells were likewise detected in C57BL/6 mice, albeit at lower frequencies. As disease unfolded in NOD mice, anti-PRPH B cells invaded the islets and increased in number at the peritoneum of diabetic but not prediabetic mice. Isotype-switched B cells were only detected in the peritoneum. Anti-PRPH B cells represent a heterogeneous population composed of both B1 and B2 subsets. In the spleen, anti-PRPH B cell were predominantly in the follicular subset. Therefore, anti-PRPH B cells represent a heterogeneous population that is generated early in life but proliferates as diabetes is established. These findings on the temporal and spatial progression of autoreactive B cells should be relevant for our understanding of B cell function in diabetes pathogenesis. The Journal of Immunology, 2014, 192: 3080–3090.

B cells are important components of the immune system that assure adequate defense against pathogens in vertebrates. However, their dysregulation can cause autoimmune diseases, being well documented, for example, in the case of lupus erythematosus where the generation of autoantibodies is the primary cause leading to pathology (1). B cells are also implied in autoimmune diseases such as type 1 diabetes (T1D) that has long been thought to be primarily dictated by autoreactive T cells that infiltrate pancreatic islets and selectively destroy insulin-producing β cells (2, 3). In NOD mice, to date one of the best murine models to study the natural pathogenesis of T1D without the necessity of artificial manipulation, the disease is highly dependent on the presence of B cells, as it is prevented through their elimination by homozygous disruption of membrane Ig-μ (4, 5).

How exactly B cells contribute to the pathogenesis of T1D is still an unsolved question. Ag specificity is key in this process. The reintroduction of transgenes encoding for a BCR that recognizes the xenoantigen hen egg lysozyme into the NOD.μ−/− background does not restore diabetes in these mice (6); however, the presence of a transgenic insulin-reactive BCR not only restores T1D but also accelerates it (7). The genesis of autoreactive B cells, their tissue distribution, as well as the exact site where they might impinge on autoreactive T cell activation have remained unknown. It is uncertain whether autoreactive B cells play a role in the initiation of the disease and are implicated in early T cell activation and proliferation, for example, in the pancreatic draining lymph nodes (LNs), or whether they are rather instrumental in the final steps of β cell destruction in the islets (8). It has been difficult to track these cells in vivo in wild-type animals owing to their presumed low frequencies and their low BCR affinities to cognate Ags. Thus, studies of these cells have been confined essentially to BCR transgenic mice.

A recent approach to assess the Ag specificities of B cells that might be implicated in T1D has been the generation of hybridomas using B cells present within infiltrated pancreatic islets of NOD mice and related insulitis-prone strains. About half of the B cell hybrids generated in this study recognized the peripheral nervous tissue (9). It was subsequently suggested that all neuronal-reactive B cells recognized the C-terminal portion of peripherin (PRPH), a cytoskeleton class III intermediate filament protein expressed in neuroendocrine tissues (10). These findings confirmed previous observations of anti-PRPH Abs in NOD mice (11, 12). In humans, anti-PRPH Abs were detected in patients with autoimmune neuropathies and endocrinopathies, but not in T1D patients without...
accompanying neurologic disorders or in healthy subjects (13). A different group found anti-PRPH Abs in humans and animals independent of T1D; however, the authors reported increased serum titers in the NOD strain compared with other, diabetes-resistant mouse strains (14).

Murine PRPH is expressed in at least four different isoforms of 61, 58, and 56 kDa (hereafter termed PRPH 61, PRPH 58, and PRPH 56, respectively) and 45 kDa (15, 16). With a single exception, in the study of Verduguer and colleagues (10) all anti-PRPH B cell hybridomas originating from islet-infiltrating B cells from NOD and related mouse strains recognize PRPH 61 and PRPH 58, but not PRPH 56. All three isoforms are composed of an N-terminal head, a central rod, as well as a C-terminal tail sequence. Whereas PRPH 61 and PRPH 58 share an identical tail sequence, replacement of the last 21 aa by an alternate eight-residue sequence is characteristic for PRPH 56. Apart from this variation, PRPH 58 and PRPH 56 are identical in their sequences. In combination, these data suggested, but did not definitively demonstrate, that the recognized epitope was either entirely or partially located in the C-terminal tail sequence of PRPH 61 and 58.

In this study, we have identified the epitope recognized by PRPH-specific, islet-infiltrating B cells. We show that the anti-PRPH B cell response is essentially confined to a single linear epitope in NOD mice. Using a novel approach, that is, the octamer display of this epitope, we have tracked anti-PRPH B cells and show that these cells segregate into different subpopulations in a tissue-specific manner. Surprisingly, anti-PRPH B cells preferentially accumulate in the peritoneal cavity even before insulitis is initiated and further increase in frequency especially in diabetic animals, in parallel with their penetration into the islets. These findings set the basis to further study peritoneal B cells, as they might be implicated in diabetes pathogenesis. The octamer approach should provide a valuable tool to search for additional B cell specificities in diabetes pathogenesis.

Materials and Methods

Reagents

For molecular cloning, enzymes were obtained from Fermentas (Madrid, Spain). Protein purification was performed using Ni-NTA–Sepharose and glutathione-Sepharose obtained from Amersham Biosciences (Barcelona, Spain). All Abs were purchased from BioLegend (San Diego, CA), Invitrogen (Barcelona, Spain), or BD Pharmingen (San Diego, CA). Streptavidin-PE and - alkalocytoxin were obtained from Columbia Biosciences (Columbia, MD). Cell culture media were obtained from Lonza (Barcelona, Spain). Peptides PRPH_{475–491} and PRPH_{435–449} generated by Fmoc solid-phase synthesis exceeded 95% purity and were obtained from GL Biochem (Shanghai, China). Unless otherwise mentioned, all other reagents were obtained from Sigma-Aldrich (Madrid, Spain).

Mice and immunizations

NOD/LtJ and NOR mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and further bred in our specific pathogen-free animal facilities. C57BL/6 mice were obtained from Charles River Laboratories (Sпain).

To test the humoral or cellular B cell response to PRPH, NOD/LtJ mice were immunized i.p. or s.c. at 6–12 wk of age with 100 μg recombinant PRPH_{292–507} emulsified in CFA (Sigma-Aldrich) or CFA only as a control.

Generation of recombinant PRPH peptides

The cDNA encoding PRPH 61 was amplified by PCR to generate recombinant peptides as indicated (Fig. 1, Table I). Peptides were cloned into a modified pGEX vector (pAκ1) (17) between the NcoI and XbaI sites. The final constructs coded for an N-terminal GST portion fused to the peptide and an extended C-terminal hexahistidine tail. Recombinant peptides were expressed in Escherichia coli strain BL21. Unless otherwise specified, recombinant fragments were purified in principle by sequential affinity chromatography using glutathione-Sepharose followed by Ni-NTA–Sepharose as published (18). Purified protein was stored in PBS under addition of 150 mM NaCl at ~80°C.

Generation of Aβ/PRPH_{475–507}C octamers

A detailed procedure for the generation of Aβ/PRPH_{475–507}C octamers will be published elsewhere (N.G., M.P., C.M., J.V. and T.S., manuscript in preparation). In brief, soluble Aβ/ molecules tethered to the N-terminal glucose-6-phosphate isomerase (GPI) peptide 282–292 (Aβ/GPI) (19) were used as scaffold for the expression and presentation of peptide PRPH_{475–507}.

The α- and β-chains of Aβ were modified C-terminally by addition of PRPH_{475–507}, followed by the extended hexahistidine tail as for the GST fusion proteins. The α-chain was further modified by inserting a site-specific biotinylation sequence no. 85 (20) between the PRPH and the hexahistidine tail. Constructs were transfected into Drosophila melanogaster–derived SC2 cells along with a vector conferring puromycin resistance. Protein was expressed using stable cell lines and purified from culture supernatants by metal chelate chromatography, followed by size exclusion chromatography as published (21, 22). Purified protein was biotinylated using the BirA enzyme as published (19). Biotinylation was measured by depletion on avidin-coated agarose beads (Pierce, Rockford, IL), followed by SDS-PAGE analysis. Aβ/PRPH_{475–507}C octamers were generated by incubation of the modified Aβ dimers with PE- or alkalocytoxin-labeled streptavidin in a 1:5 molar ratio.

Islet isolation

Pancreatic islets from NOD females were isolated using a collagenase infusion method (23) and further handpicked into HBSS under a dissecting microscope before overnight culture.

Cell staining and flow cytometry analysis

Single-cell suspensions of spleen, bone marrow, and LN’s were generated by mechanical disruption of the corresponding organs in PBS/2% FCS. Peritoneal cells were obtained by flushing the peritoneal cavity with cold solution of PBS/2% FCS. Erythrocytes were removed by lysis. Ag-specific B cell analysis was carried out using PE- or alkalocytoxin-labeled Aβ/PRPH_{475–507}C octamers or Aβ/GPI tetramers as negative control. Cells were incubated with octamers at a final concentration of 8.5 μg/ml in FACS buffer (PBS/5% FCS) for 15 min on ice. For inhibition studies, Aβ/PRPH_{475–507}C octamers were preincubated overnight with mAb 228 E1 at 4°C before cell staining (10). For PRPH B cell distribution studies and costaining of additional cell surface markers of islet-resident B cells, FITC-labeled anti-B220 and CD19 as well as PE-C5y-labeled anti-CD3, anti-CD11c, and anti-F4/80 (dump channel) were used. Exclusion of dead cells was done by addition of 5 μg/ml propidium iodide immediately before acquisition. For activation marker analysis, cells were costained using Aβ/PRPH_{475–507}C octamers labeled with alkalocytoxin as well as with PE (double staining approach) as mentioned above; however, alkalocytoxin-Cy7–labeled anti-CD19, Pacific Blue–labeled anti-CD86, or anti-CD44 were used in addition to the mentioned PE-C5y-labeled Abs for the dump channel. Follicular/marginal zone (MZ) and IgM/IgD PRPH C costaining was carried out by the octamer double staining approach together with FITC-labeled anti-CD21 or anti-IgM, Pacific Blue–labeled anti-CD21, or anti-IgD as well as alkalocytoxin-Cy7–labeled anti-CD19 and PE-C5y–labeled anti-CD3, anti-CD11c, and anti-F4/80 anti-CD3 and propidium iodide. Data acquisition was performed using FACScan, FACSCalibur, FACSCanto II, and LSR II instruments (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

B cell isolation and activation

B cells were isolated from the respective organs and purified to 97% by positive selection using anti-CD19 Ab-coated MACS MicroBeads according to the manufacturer’s specifications (Miltenyi Biotec, Cologne, Germany). Peritoneal cells were enriched for B cells by removal of adherent cells through incubation during 2 h at 37°C in complete RPMI 1640 media containing 10% FCS, 50 μU/ml penicillin, 50 μg/ml streptomycin, 1 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME. Purity of CD19+ lymphocytes was >80%. Pancreatic islets were isolated by collagenase digestion of perfused pancreata as described (24).

For activation, enriched total CD19+ B cells and pancreatic islets were resuspended in complete RPMI 1640 media. B cells were cultured at a density of 5 × 10^6 cells/well in 24-well plates in 1 ml; pancreatic islet were seeded into 96-well plates using 250 μl final volume and 20 islets/well. For activation, LPS was added at 10 μg/ml. After 14 d, culture supernatants were collected to test Ig secretion by an isotype-specific ELISA.
Western and dot blot analysis
For dot blot analysis, 1 μg protein was directly spotted on a nitrocellulose membrane. For Western blot analysis, 5 μg purified protein or total E. coli cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in 10 mM Tris, 0.1 M NaCl, pH 7.5, with 0.1% Tween 20 for 2 h at room temperature (TBS-B), incubated overnight at 4 °C with sera or hybridoma culture supernatants diluted in TBS-B, and washed. Bound IgS were detected by incubation with HRP-conjugated goat anti-mouse IgG (H+L) or goat anti-mouse IgM (both from BioLegend), followed by avidin-HRP (eBioscience). Binding of secondary Abs was revealed through chemiluminescence using a Fujifilm LAS-3000 system (R&D Systems).

ELISPOT assay
Total splenocytes and peritoneal cells were costained with allophycocyanin-labeled Ab PRPH429–507, Cy5-labeled Ab A1, and PE-Cy5-labeled Ab PRPH467–507 in supernatants as well as FITC anti-CD19, PE-Cy5 anti-CD3, anti-F4/80, and anti-CD11c Abs. Labeled cells were sorted using a FACS Aria cell sorter (Becton Dickinson). ELISPOT plates (nitrocellulose-bottomed 96-well MultiScreen-HA filtration plates; Millipore) were coated with 80 μg/ml PRPH429–507 fused to GST or GST only and incubated at 4 °C overnight. Plates were washed three times with wash buffer (PBS/0.1% Tween 20) and blocked with 3% bovine serum albumin for 1 h at 37 °C. Plates were incubated for 72 h at 37 °C. Wells were washed three times and incubated for 2 h at room temperature with biotin-conjugated goat anti-mouse IgG (eBioscience) and developed by adding 3-amino-9-ethylcarbazole substrate. To stop the reaction, plates were washed with water. The number of spots was evaluated using an AID ELISPOT reader.

ELISA and ELISA competition
MaxiSorp flat-bottomed plates (Nunc) were coated with 10 μg/ml Ag overnight at 4 °C. Whenever GST fusion proteins were used as coating Ags, GST protein lacking the PRPH peptide was used to establish blank controls. For comparative analysis of FRPH fragments varying in length, equal dilutions of the GST fusion proteins, A 2 g7/PRPH 467–507, were used as negative controls as indicated for Western blot analysis. For ELISA competition, sera or culture supernatants diluted in TBS-B, and washed. Bound Igs were detected by incubation with HRP-conjugated goat anti-mouse IgG (H+L) or goat anti-mouse IgM (both from BioLegend), followed by avidin-HRP (eBioscience). For intraexperimental controls, the exception of the N-terminal fragment, PRPH1–84, which was consistently expressed in a truncated form (Fig. 1A). Western blot analysis confirmed recognition of the last C-terminal 79 aa (PRPH129–197) by mAb I6, 1 of >20 anti-PRPH mAbs previously generated by the Verdaguer Laboratory (9, 10) that had been randomly selected for this study. To further narrow down the epitope, three additional overlapping peptides of the C terminus were generated, of which only the one containing the last 41 aa (PRPH467–507) was recognized by the mAb (Table I). Further analysis of N- as well as C-terminal truncated versions of this peptide revealed a consensus sequence of 17 aa, PRPH475–491, recognized by mAb I6 (Fig. 1B, Table I). As shown in Table I, the last 6 aa of this epitope are missing in PRPH 56 owing to alternate splicing, explaining the loss of recognition of this isoform by the anti-PRPH mAbs analyzed in the previous study (10).

To establish whether recognition of the PRPH475–491 epitope was limited to mAb I6, or common to most anti-PRPH B cell hybridomas, we analyzed the reactivity of 14 additional, randomly selected B cell hybridomas from our collection by dot blot. Indeed, all mAbs recognized PRPH475–491 (not shown), indicating that this epitope is a major or even the sole epitope recognized by islet-infiltrating anti-PRPH B cells. A previous analysis of the variable BCR regions of the anti-PRPH B cell hybrids revealed that these clones were all independent, with some of them probably deriving directly from the preimmune repertoire with others showing clear signs of somatic hypermutation (25). Although all mAbs derived from these clones reacted with PRPH475–491, the optimal epitope might differ between the individual B cells. Indeed, a comparative ELISA analysis of four PRPH fragments containing the 475–491 epitope but differing in length owing to adjacent N- and C-terminal amino acids showed that the analyzed mAbs had different affinities for these fragments. Unexpectedly, but possibly due to steric hindrance, the longest fragment was not always best recognized (Fig. 1C).

A nonconformational-dependent PRPH epitope is recognized by islet-infiltrating B cells
The PRPH target Ag of islet-infiltrating B cells was detected by analysis of cell lysates under denaturing conditions (10). Similarly, to carry out the epitope mapping, GST fusion proteins were denatured by heating prior to SDS-PAGE and Western blot analysis. Epitope recognition under these conditions argued for the epitope to be nonconformational. To further test this possibility, we carried out a dot blot analysis using a selection of recombinant PRPH-derived peptides that either had been denatured by heating or had been left untreated. The I6 hybridoma chosen for this analysis recognized the epitope both in the untreated or treated form (not shown). Similarly, treatment with urea of PRPH429–507 did not alter its recognition by I6 when tested by ELISA (Fig. 1D). A synthetic version of peptide PRPH475–491 was able to partially prevent binding of I6 by competition to plate-bound PRPH429–507 (not shown). Inhibition assays using a longer peptide, PRPH473–495, which was recognized with higher affinity according to the ELISA shown in Fig. 1D, inhibited three randomly selected anti-PRPH
mAbs binding to 100% at 1 mM (Fig. 1E, Supplemental Fig. 2A). PRPH473–495 was also able to completely block binding of mAb 228 E1, the Ab used previously by us to identify PRPH as an autoantigen (10), to native PRPH expressed by the neuroblastoma N1E-115 (Supplemental Fig. 2B). Taken together, the results confirmed that PRPH475–491 was the minimal linear epitope recognized, although longer epitopes confer stronger Ab binding.

Anti-PRPH humoral response in insulitis-prone and insulitis-resistant mouse strains

We further investigated how the response to the C-terminal portion of PRPH evolved in the diabetes-prone NOD mouse compared with the genetically closely related but diabetes-resistant NOR, (NOD × NOR) F1, as well as C57BL/6 strains. Whereas (NOD × NOR) F1 mice develop insulitis, this pathology is negligible in NOR mice and completely absent in C57BL/6 mice. Sera of 3- to 31-wk-old NOD mice were analyzed by ELISA for Abs against PRPH429–507. PRPH-specific Abs were detected in all strains (Fig. 2). However, the response was delayed and less pronounced in C57BL/6 mice. These data indicated that although neither insulin nor diabetes was a requirement for the generation of anti-PRPH Abs, genes related to the NOD background were possibly implicated in this accelerated response. In all strains, at ages of 20 and 31 wk, the anti-PRPH response was more pronounced in females than in males.
The anti-PRPH response in the NOD mouse is focused to the C terminus

All B cell hybridomas analyzed in this study that had been isolated from pancreatic islets recognized the C terminus of PRPH 61 and PRPH 58. This prompted us to analyze whether this was an islet-restricted phenomenon, or whether the C-terminal response was systemic. Of the seven overlapping fragments (covering PRPH85 to PRPH507) analyzed by Western blot (not shown) or ELISA, only PRPH429–507 was clearly recognized by sera from 5- (not shown) and 24-wk-old NOD females. Of the two Ig classes analyzed, IgG was clearly detected, whereas the IgM response was much less pronounced (Fig. 3A, 3B). To test the extent to which the in vivo response was directed against the PRPH473–495 epitope, we carried out a competition ELISA using NOD sera. The synthetic peptide PRPH 473–495 inhibited polyclonal Ab binding to plate-bound PRPH473–495 by ∼80% at high concentration (1 mM; Fig. 3C). This result indicated that a sizable proportion of the anti-PRPH response was directed against PRPH473–495. However, based on the high peptide concentration needed for competition, the Abs were likely of low affinity. Bearing in mind the caveats that the first 84 aa were incompletely analyzed, as PRPH1–84 did not express at full length in E. coli, and that only nonconformational peptides or peptides of unconfirmed conformations of PRPH were scanned in these assays, these results suggest that a dominant and possibly exclusive response was generated against the C terminus of PRPH. Because Abs against PRPH429–507 were detected as early as 3 wk of age in NOD mice when these mice are still free of insulitis, this argued for a response originating in tissues different from pancreatic islets.

A weak boost response upon immunization with PRPH429–507

Because the anti-PRPH response was present in mouse strains that develop neither insulitis nor diabetes and was detectable as early as

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**Table 1. Mapping of the epitope recognized by islet-infiltrating anti-PRPH B cells**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Recognition by I6 Hybridoma</th>
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<tbody>
<tr>
<td>430–440</td>
<td>+</td>
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<td>440–450</td>
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This amino acid sequence of the GST-PRPH-6H fusion proteins corresponding to PRPH only is shown. Consensus sequence is in bold; partial epitope of PRPH 56 is in bold and italics.

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**FIGURE 2.** Anti-PRPH humoral response in diabetes-prone and diabetes-resistant strains. ELISA detection of anti-PRPH IgG in sera harvested from NOD, NOR, (NOD × NOR) F1, hybrids, and C57BL/6 mice. For detection, PRPH429–507 was coated to plates. Relative unit (RU) values were normalized to an internal anti-hexahistidine Ab control run in each experiment to allow cross-plate comparisons of data. Sera dilution was 1:300. Data are the means ± SD of three to five mice in each group. The *p value was determined by a one-way ANOVA test for each female and male group. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 3. IgG, but not IgM, anti-PRPH response in NOD mice. (A) ELISA detection of anti-PRPH IgG in sera harvested from 24-wk-old NOD mice. For detection, overlapping peptides covering PRPH435–449 (compare with Fig. 1) were coated to plates. Bound IgG was detected using anti-mouse IgG. Values of the GST blank control were subtracted during analysis. Data (mean values ± SD) are representative of three independent experiments. The p value was determined by a one-way ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001, comparing Abs binding to PRPH peptides with the GST control. (B) ELISA detection of anti-PRPH IgM in sera harvested from 24-wk-old NOD mice. The assay was carried out as in (A) with the difference that bound IgM was revealed using an anti-IgM Ab. Values of the GST blank control were subtracted during analysis. Data (mean values ± SD) are representative of three independent experiments. The p value was determined by a one-way ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001, comparing Ab binding to PRPH peptides with the GST control. (C) Detection of Abs specific for the synthetic PRPH473–495 peptide in NOD sera. An ELISA competition assay is shown using plate-bound PRPH473–495 and free, synthetic PRPH473–495 for competition. Sera from 24-wk-old NOD females were preincubated using the indicated concentrations of free peptide overnight and next added to coated plates. Bound mAb was detected as in (A). The irrelevant synthetic PRPH435–449 peptide was used as a control in this assay. (D) ELISA detection of PRPH467–507-specific IgG in sera from NOD females after immunization with two doses of 100 μg PRPH429–507 at days 0 and 21 emulsified in CFA and IFA, respectively. Age-matched (12-wk-old) naive NOD females served as controls. Recognition of plate-bound PRPH467–507 was analyzed. Specific IgG was detected as in (A); values of the GST blank control were subtracted during analysis. Sera dilution was 1:300 unless indicated otherwise in the figure. Mean values ± SD of from five mice per group are shown. The p value was determined by a two-way ANOVA test. ***p < 0.001, comparing immunized with naive control mice.

In vivo detection of PRPH-specific B cells by AEl/PRPH467–507C octamers

To analyze the genesis of the anti-PRPH response, we searched for Ag-specific B cells in vivo using fluorescently labeled peptide. However, several conventional approaches, such as FITC labeling of synthetic peptide, peptide coating of fluorescent beads, biotinylated GST peptide fusion proteins, or biotinylated synthetic peptide, both incubated with PE-labeled streptavidin, failed to detect PRPH-specific B cells. The GST fusion protein stained B cells nonspecifically, and the synthetic peptide did not substantially stain I6 (not shown), the sole hybridoma of islet-infiltrating anti-PRPH B cells of our collection that expressed surface IgG. We argued that this problem might be overcome by increasing the peptide valency. To do so, we used the intrinsic heterodimeric nature of MHC class II molecules and fused PRPH467–507 to the C terminus of the α- and the β-chain of soluble, recombinant AEl molecules, the sole MHC class II variant expressed by the NOD mouse. PRPH467–507 rather than PRPH435–449 was used to generate these constructs because, overall, this peptide was best recognized by all hybridomas analyzed. AEl heterodimeric molecules were further stabilized by fusion of a peptide (GPI) at the N terminus of the β-chain that folds into the peptide-binding groove (19). In this format, the AEl/GPI molecule served as scaffold to facilitate expression and purification of the PRPH peptides. Site-specific biotinylation at the C terminus of the AEl α-chain and incubation of these fusion proteins (AEI/PRPH467–507C) with fluorescently labeled streptavidin leads to the octameric display of the peptide (Fig. 4A).

We first verified the specificity of the reagent by assessing its ability to stain clone I6. The B cell hybrid was stained by an anti-IgG Ab as well as by AEl/PRPH467–507C octamers, but not by the AEl control scaffold reagent that lacked PRPH467–507 (tetramers of AEl/GPI; Fig. 4B). The BCR was key for staining by the octamers, as a pre–B cell line lacking surface Ig was not stained (not shown). The staining was peptide specific as it could be blocked by pre-incubation of the AEl/PRPH467–507C octamers with the anti-PRPH mAb 228E1 (Fig. 4B). When spleens from 7-wk-old NOD females were analyzed (Fig. 4C), on average 0.19% of B cells were stained by AEl/PRPH467–507C octamers compared with 0.1% by the AEl GPI tetramer control, suggesting that ~0.09% of B cell were specific for PRPH467–507 and demonstrating that the octamers did not stain all B cells indistinguishably.

An Ab response against AEl in the NOD mouse has been suggested previously, and B cells of this specificity therefore should recognize and thus might be stained by Ag7/PRPH467–507C octamers as well (11). Additionally, the analysis of rare Ag-specific B cells is hampered by the presence of B cells that recognize PE (26). B cell staining by our control reagent also suggested that some recognition irrelevant to the PRPH peptide occurred. To minimize nonspecific B cell detection, we generated AEl/PRPH467–507C octamers labeled with either PE or allophycocyanin and incubated peritoneal cells with both octamers simultaneously. In parallel, peritoneal cells were incubated with control AEl/GPI tetramers prepared with the same fluorochromes. If, on the one hand, AEl-specific B cells were present and stained by the octamer, they should also be stained by the tetramer control, owing to the identical AEl scaffold. On the other hand, the double color approach should serve to eliminate either allophycocyanin- or PE-specific B cells, as those cells would be stained by either the PE- or the allophycocyanin-containing reagent only, but not by both simultaneously. As shown in Fig. 4D, AEl-specific B cells were essentially absent or not stained, as no double-positive cells using the tetramer control reagent became apparent. On the contrary, ~0.25% of B cells were detected by the double color octamer staining approach, alleviating concerns of

3 wk after birth, it possibly represented a natural Ab response. Such responses cannot be boosted upon immunization with Ag and are primarily made up by IgM Abs. To test whether the response to PRPH increased upon immunization, we injected into NOD females recombinant PRPH429–507 emulsified in CFA, boosted after 3 wk using the same protein emulsified in IFA and analyzed the specific Ab response 4 d later by ELISA. Immunization with GST only served as control. Animals mounted a strong humoral response to GST, indicating that anti-GST B cells were fully responsive (Supplemental Fig. 3). Conversely, the response to PRPH467–507 was substantially weaker (Fig. 3D). In conclusion and in light of the lacking IgM response mentioned earlier, the comparatively weak boost response pointed toward a low-affinity Ab response.
single PE- or allophycocyanin-positive B cells. Therefore, the two-color approach served to detect PRPH-specific B cells only (hereafter referred to as PRPHC B cells).

As a final proof of the suitability of the \( \text{Ag}^7/\text{PRPH}_{467-507} \) octamer for PRPHC B cell identification, we applied the double color octamer staining approach to sort B cells and to analyze their specificity by ELISPOT. Four thousand octamer+ or total CD19+octamer B cells were sorted into plates coated with GST-PRPH_{429-507} or with GST only, stimulated with anti-CD40 Ab, and secreted Abs were detected with an anti-mouse \( \kappa \) Ab (Fig. 4E). For comparison, 1000 I6 hybridoma cells were seeded into coated plates as parallel experiments showed that after the 3-d incubation period, a similar cell number was obtained in the three cultures due to the strong proliferation of the hybridoma. Similar amounts of spots were identified in the well containing either PRPH C B cells or I6 hybridoma cells, whereas CD19+octamer B cell–containing wells were undistinguishable from wells containing medium only. The presence of PRPH-specific Ab secretion was also evidenced by a darker color of the membrane found exclusively in wells that had received either octamer+ B cells or I6 hybrids and that had been coated with PRPH_{429-507}. Neither I6 nor PRPHC B cells recognized coated GST. Collectively, these results conclusively validated \( \text{Ag}^7/\text{PRPH}_{467-507} \) octamers as a tool to detect PRPHC B cells.
**In vivo distribution of PRPH<sub>C</sub>B cells**

We used the A<sup>67</sup>/PRPH<sub>467–507</sub>C octamers to analyze the distribution of PRPH<sub>C</sub>B cells in vivo in NOD mice (Figs. 4C, 5A). PRPH<sub>C</sub>B cells were detected in the spleen, LNs (only pancreatic LNs are shown), as well as in the peritoneum at ages between 4 and 15 wk. Overall, more PRPH<sub>C</sub>B cells were detected in females compared with males. Unexpectedly, the highest percentages of PRPH<sub>C</sub>B cells were found in the peritoneum with up to 0.5% of total B cells in females. The peritoneum and pancreatic LN PRPH<sub>C</sub>B cells progressively increased in females where they peaked at week 15, the oldest age analyzed. We wondered whether the increment of PRPH<sub>C</sub>B cells correlated with disease progression. Indeed, a separate set of experiments revealed that the peritoneum of 15-wk-old diabetic NOD females contained significantly more PRPH<sub>C</sub>B cells than did age-matched, nondiabetic individuals (Fig. 5B). In pancreatic islets, PRPH<sub>C</sub>B cells were undetectable at 5 wk of age, but at 10 and 15 wk of age, ~0.2% in infiltrating B cells stained with the octamers (Fig. 5C, only 15-wk-old mice are shown). These results suggested that the primary site of PRPH<sub>C</sub>B cell proliferation might not be the pancreas or pancreatic islets but rather the peritoneum. Alternatively, PRPH<sub>C</sub>B cells may preferentially home to the peritoneum. A third explanation for the dearth of PRPH<sub>C</sub>B cells in pancreatic islets could be their rapid conversion at this site into a plasma cell–like phenotype that would escape detection by the octamer due to downregulation of the BCR as previously shown (Ref. 27 and N.G., not shown). Further support for this idea was the detection of anti-PRPH Abs in islet culture supernatants (not shown).

To establish whether PRPH<sub>C</sub>B cells were augmented in diabetes-prone NOD mice compared with disease-resistant C57BL/6 mice, we analyzed 7-wk-old females from both strains side-by-side. Although present in both strains, PRPH<sub>C</sub>B cells were significantly elevated in females of the NOD compared with the C57BL/6 strain in spleen and peritoneum (Fig. 5D).

**Tissue-dependent phenotypic variations of PRPH<sub>C</sub>B cells**

The presence of PRPH<sub>C</sub>B cells in the peritoneum was a further indication that at least a portion of these cells might belong to the natural B-1 B cell compartment. Approximately 30% of PRPH<sub>C</sub>B cells expressed CD5 in the peritoneum (Fig. 6A, top panels), indicating that these cells belonged to the B-1a subtype. Additionally, 15% of PRPH<sub>C</sub>B cells were stained by Mac-1 but not by CD5, a hallmark of B-1b B cells (28), which was close to triple the...
percentage detected in total CD19+ B cells at the same site. In the spleen and pancreatic LNs, most PRPHC B cells were CD5−Mac1+. Whereas percentages, owing to the relatively low cell number, are susceptible to fluctuation, the results nevertheless mirrored the percentages of total CD19+ B cells at these sites and clearly demonstrated that PRPHC B cells are a heterogeneous population. Costaining with anti-IgM and anti-IgD revealed that in all sites the majority of PRPHC B cells were either IgD+ or IgM−IgD+, very similar to total CD19+ B cells (Fig. 6A, bottom panels). However, some switching to IgG, the isotype expressed by virtually all PRPHC B cell hybridomas derived from pancreatic islets (25), might be supported in the peritoneum, the only site where IgM−IgD− cells among this population were observed. On the contrary, ~10% of IgM−IgD− cells were found at all sites within the total CD19+ B cell population. In infiltrated islets, B-1a and B-1b B cell were identified in CD19+ total, but not in PRPHC B cells (Fig. 6B). Contrary to total B cells where ~30% had switched their isotype based on absence of IgM/IgD expression (3-fold more than found in spleen, pancreatic LNs, or peritoneum), IgM−IgD− PRPHC B cells were essentially absent at this site.

Finally, we analyzed the phenotype of PRPHC B cells in the spleen by costaining with anti-CD21 and anti-CD23 Abs (Fig. 6C). At this site, ~50% of PRPHC B cells were double positive for these markers, a typical phenotype of follicular B cells, whereas ~15% of PRPHC B cells resembled MZ B cells (CD21hiCD23lo). This was comparable to the frequencies detected in the total B cell population and a further confirmation of the heterogeneity of anti-PRPHC B cells.

**Discussion**

The implication of B cells in T1D has long been recognized. Humoral responses toward autoantigens such as insulin or glutamic acid decarboxylase were discovered several decades ago, and autoantibodies have served to establish the diagnosis of T1D risk in the clinic (29, 30). The study of the corresponding cellular responses, however, has proven much more cumbersome. Cyto- metric detection of Ag-specific B cells by increasing the valency of epitopes has been reported for BCR transgenic or immunized mice, using either random coupling to a fluorescent carrier molecule or tetramerization of the biotinylated Ag (31–33). The detection of autoreactive B cells in nontransgenic naive mice or patients using similar methods either failed or was not reported to our knowledge (7, 34). Especially in wild-type NOD mice, the identification of autoreactive B cells that recognize natural autoantigens has been hampered by the low affinities of autoreactive BCRs for their cognate Ags (35). However, recent achievements to prevent T1D by targeting Ag-specific B cells call for novel approaches to be able to track these cells for preclinical and clinical studies (36). The previously suggested presence of PRPH-specific B cells in pancreatic islets (9, 10) prompted us to conduct an in-depth analysis of this B cell reactivity using a novel approach consisting of the octameric display of the cognate Ag and to search for the sites of autoreactive B cell accumulation.

The first relevant finding in the present study was that all PRPH-specific B cells recognized the same core epitope, although, as expected owing to the BCR variants previously described (25), their fine specificities were different. Our findings also indicate a dominant response to this epitope in young animals that is maintained at least until 24 wk of age, the oldest age that was analyzed in a side-by-side comparison of the seven expressed fragments covering the 61-kDa isoform of PRPH25–507. Do other epitopes of a similar significance exist that we may have overlooked in our study? We cannot completely exclude this for several reasons, including the partially missing N-terminal 84 aa that eluded our analysis as well as the mentioned limitation that we only analyzed protein fragments of unknown tertiary structure. Furthermore, some epitopes close to the fragment boundaries might have been lost. However, albeit PRPH as a target Ag of islet-infiltrating
B cells has been detected using denaturizing conditions (10), the neuronal recognition pattern of all clones had been carried out using air-dried cryosections (9), a technique that can preserve three-dimensional protein structures. If, at least in case of the islet, a dominant B cell population recognizing a different epitope of PRPH was present at this site, it is likely that this population would form part of the hybridoma collection previously generated. However, all hybrids that we analyzed recognized the C-terminal epitope. The humoral response toward the PRPH C terminus was not restricted to the NOD strain but was also found in other NOD-related and unrelated strains. In the four strains analyzed, the response was strongest in C57BL/6 mice and delayed compared with other strains. In all strains, the response was more pronounced in females. Although the function of anti-PRPH B cells still needs to be established, it is tempting to speculate that NOD background-related genes might be involved in the increase of this cell specificity.

The second noteworthy finding, facilitated by the octamer approach, was the tissue distribution of PRPH C B cells. The highest percentages of these cells were found in the peritoneum. However, these cells were not limited to the NOD strain, but were also found, albeit at lower levels, in C57BL/6 mice, as expected from our data demonstrating the existence of anti-PRPH Abs in this strain. The high percentages of PRPH C B cells in the peritoneal cavity combined with the low Abs affinities detected by ELISA inhibition and the comparatively weak Abs response upon immunization were indicative that these cells may form part of the natural B cell compartment or produce low-affinity Abs (37). The CD5/Mac-1 analysis indicated that PRPH C B cells did not form a homogeneous B cell population. In the peritoneum, ~50% of these cells belonged either to the B-1a or the B-1b population, but the vast majority of PRPH C B cells in pancreatic LNs and spleen were CD5−, indicating that they were either follicular B cells, MZ B cells, or possibly in part B-1b cells that are CD5− and do not express Mac-1 outside the peritoneum (28, 38). A BCR analysis should reveal whether the B-1 peritoneal population is of different clonal origin that may explain the heterogeneity of PRPH C B cells. B-1 cells have been implied previously to play a role in T1D pathogenesis. Hypotonic lysis leading to B-1a or B-1b cell depletion in the peritoneum leads to a delayed disease onset in NOD mice (39). Very recently, its has been suggested that the possible role of B-1 cells in T1D is to promote autoreactive T cell access to pancreatic islets, possibly by the induction of VCAM-1 expression on the pancreatic vasculature (40). It is thus possible that some of the PRPH C B cells are involved in a similar process. PRPH C B cells in the islet were negative for Mac-1 and CD5; however, it is possible that these markers were lost during the overnight culture process. Coanalysis of CD21 versus CD23 expression revealed that PRPH C B cells were not limited to the B-1 B cell compartment. Rather, we detected tissue-specific differences: in the spleen, most PRPH C B cells resembled follicular B cells, yet ~15% could also be classified as MZ B cells. MZ B cells have recently been described to invade pancreatic islets and to have the capacity to present autoantigens such as insulin, leading to T cell activation in vitro (41). We have not yet established whether some PRPH C B cells in the islets could be classified as MZ B cells.

A further question remaining to be solved is whether and where these cells are implicated in Ag presentation. A recent report has shown that even tolerant anergized B cells are fully capable of Ag presentation and T cell activation (42). Ag presentation could occur in the peritoneum or in pancreatic LNs, besides others. It has previously been suggested that pancreatic LNs are the critical site of islet Ag–specific T cell priming to condition their capacity to infiltrate the islets (43), and the removal of these LNs led to an almost complete protection against insulitis and diabetes (44).

Passive or active transport of PRPH from the pancreas released during physiological B cell death (43) could be a source of Ag for PRPH C B cells that is subsequently presented to the corresponding T cells. However, PRPH C B cells may also pick up Ag or simply get activated in the peritoneum and then home directly to pancreatic LNs, a mechanism previously described (45). In females, as the disease progressed, the increment of PRPH C B cells in the peritoneum was paralleled by an increment of these cells in pancreatic LNs. Whether this is due to proliferation in one of these sites only, followed by trafficking, or whether these populations proliferate or simply accumulate at different sites independently remains to be established.

As mentioned previously, islet-infiltrating PRPH C B cells secreted IgG, and in the present study, serum IgM levels were only marginal. This begs the question where and under which conditions isotype switching is induced in NOD females. The IgM/IgD analysis suggests that it occurred neither in the spleen nor in pancreatic LNs, as essentially no IgM/IgD double-negative cells were detected at these sites. Switching might take place in the peritoneum where CD19+ IgM/IgD double-negative cells were detected. In case of the peritoneum, it is possible that omental milky spots are involved in this process, as it has recently been described that isotype switching and somatic hypermutation are supported in this tissue (46). Another possibility is that switching and affinity maturation take place in the islets themselves. However, if this is the case, it is much less pronounced compared with the total B cell population that was composed by almost a third of switched B cells. Tertiary lymphoid structures associated to pancreatic islets have been described previously (47–49), and therefore local switching events could be supported at this site.

Whether the response to PRPH is T cell–dependent is still an open issue. We are currently investigating this question. The BCR analysis of islet-infiltrating PRPH-reactive B cells showed that some, but not all, had undergone somatic hypermutation (25), a clue that argues for a T cell/B cell interaction. The rather high percentages of PRPH C B cells in the peritoneum already detectable at young age seemingly advocates against it, as does the finding that some PRPH C B cells belong to the B-1 B cell subpopulation. Because the phenotypical analysis argues for a heterogeneous B cell population, it is possible that this response is partially, but not completely, T cell–independent. T cell–independent activation of autoreactive B cells has recently been described, which is not staged in germinal centers but at the T cell zone/red pulp border (50, 51). Whether the same holds true for PRPH C B cells needs to be analyzed further. The identification of PRPH-specific autoreactive T cells, that is, by MHC tetramers, would prove invaluable to gain a deeper insight of the collaboration between both cell types in T1D pathogenesis.

The response against PRPH adds to a growing list of autoantigens derived from neuronal tissues for which a causative link to the pathogenesis of T1D has been shown (52, 53). The detection of elevated percentages of B cells especially in recent-onset diabetic animals recognizing such an Ag in the peritoneum is unexpected. It should encourage the search for further autoreactive B cell populations in the peritoneum. The octamer approach is likely to prove valuable for this endeavor.

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