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# The Bw Cells, a Novel B Cell Population Conserved in the Whole Genus *Mus*

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Jean-Marc Cavaillon,<sup>‡</sup> Pierre-André Cazenave,<sup>\*</sup> and Dominique Rueff-Juy<sup>\*§</sup>

In common laboratory mouse strains, which are derived from the crossing between three subspecies, peritoneal B cells are enriched in B-1a cells characterized by the CD5<sup>+</sup>Mac-1<sup>+</sup>B220<sup>low</sup>IgM<sup>high</sup>IgD<sup>low</sup>CD43<sup>+</sup>CD9<sup>+</sup> phenotype. Intriguingly in other vertebrates, CD5<sup>+</sup>Mac-1<sup>+</sup> cells have never been found in a specific anatomic site. To ascertain the peculiarity of the CD5<sup>+</sup> peritoneal B cells in laboratory mice, we analyzed the peritoneal B cell subsets in 9 inbred and 39 outbred wild-derived mouse strains belonging to 13 different species/subspecies. We found that most of these strains do not have the CD5<sup>+</sup> B-1a cell population. However, all of these strains including classical laboratory mouse strains, have variable proportions of a novel B cell population: Bw, which is characterized by a unique phenotype (CD5<sup>-</sup>Mac-1<sup>+</sup>B220<sup>high</sup>IgM<sup>high</sup>IgD<sup>high</sup>CD43<sup>-</sup>CD9<sup>-</sup>) and is not restricted to the peritoneal cavity. Bw cells are also distinct from both B-1 and B-2 cells from a functional point of view both by proliferative responses, cytokine secretion and Ab synthesis. Moreover, transfer experiments show that bone marrow and fetal liver cells from wild mice can give rise to Bw cells in alymphoid mice. The conservation of this B cell population, but not of the CD5<sup>+</sup> B-1a, during evolution of the genus *Mus*, its readiness to respond to TLR ligands and to produce high concentration of autoantibodies suggest that Bw cells play a key role in innate immunity. *The Journal of Immunology*, 2007, 179: 6568–6578.

In laboratory mouse strains, the mature B cell pool can be divided into two major subsets, B-1 and B-2, which differ according to anatomical location, phenotype and function (1, 2). In the spleen, mature B lymphocytes belong mainly to the conventional B-2 population, which consists of a large subset of follicular B cells (FO: B220<sup>high</sup>Mac-1<sup>-</sup>IgM<sup>low</sup>IgD<sup>high</sup>CD21<sup>low</sup>CD23<sup>high</sup>) preferentially engaged in responses to T-dependent Ags, and a discreet subset of marginal zone (MZ)<sup>3</sup> B cells (MZ B: B220<sup>high</sup>Mac-1<sup>-</sup>IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>high</sup>CD23<sup>low</sup>) more specialized in responses to T independent (TI) Ags (3). In the peritoneal cavity, B cells consist of the conventional B-2, and of a Mac-1<sup>+</sup>B220<sup>low</sup>IgM<sup>high</sup>IgD<sup>low</sup>CD9<sup>+</sup>CD43<sup>+</sup> B-1 population, which can be divided into 2 subsets according to the expression of CD5: B-1a (CD5<sup>+</sup>) and B-1b (CD5<sup>-</sup>) cells (1, 4). MZ B and B-1a cells were shown to be functionally related and to provide immediate responses against bacterial pathogens while B-1b cells would be involved later in acquired immunity (3, 5–7).

B-1a cells have long been considered as the major source of serum IgMs, natural autoantibodies and to respond mostly to TI-1 Ags (1, 8–10). They were also shown to display an oligoclonal repertoire directed against conserved structures expressed by a large subset of microorganisms or present on eukaryotic cell membranes such as phosphorylcholine (PC), phosphatidylcholine (PtC), an Ag unmasked by treatment of mouse erythrocytes with bromelain, or autoantigens such as actin or DNA (1, 11–13). B-1a commitment has long been a matter of debate (2, 14–16), although recent data bring evidence of the existence of B-1 cell-specified progenitors (17). In laboratory mouse strains, CD5 is constitutively expressed only on T and B-1a lymphocytes. In humans, CD5 is expressed mainly on cord blood B cells and on 30% of adult B lymphocytes. In rabbits and chickens, the CD5 Ag is expressed on almost all B cells (2).

Our knowledge of B cells in mice relies on studies conducted in laboratory strains established during the past century and derived from a very small pool of ancestors (18). Common laboratory strains can be considered as recombinant strains derived from 3 subspecies in unequal proportions: *Mus musculus domesticus*, *Mus musculus musculus* and *Mus musculus castaneus* (18). The vast majority of them display the same maternally inherited mitochondrial DNA from *M. musculus domesticus* and the Y chromosome from *M. musculus musculus* (19). For these reasons, their genetic polymorphism is rather limited in comparison with the whole wild-derived mouse strains. The latter were established twenty years ago from independent progenitors trapped in the wild from different regions of the world and belong to different well-defined species/subspecies. These wild-derived strains present a high level of genetic polymorphism when compared with that of laboratory strains and are supposed to reflect more closely human diversity (18).

It is now well established that common laboratory strains do not fully represent the biology of the entire genus *Mus* (18). At the immunological level, we have shown that B and T cell repertoires are very different in laboratory and wild-derived strains (20–22).

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<sup>3</sup> Abbreviations used in this paper: MZ, marginal zone; BM, bone marrow; CpG, cytosine phosphate guanosine oligodeoxynucleotide; FL, fetal liver; PC, phosphorylcholine; PtC, phosphatidylcholine.

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Additionally, the Th-1/Th-2 dichotomy described in laboratory strains (23) is not so clear-cut in wild-derived mice as exemplified by immune response to cutaneous leishmaniasis. Indeed, wild-derived mice present a gradient of diseases, some of them recapitulating more closely human diseases (24). Similar data were reported in other model of infectious disease (25).

The present study was undertaken to revisit the representativity and functional properties of the peritoneal B cell subsets among the genus *Mus*, making use of 48 wild-derived mouse strains. Our study reveals a novel B cell population, the Bw cells, found in variable proportions in peritoneal cavity, lymphoid organs and PBL of all wild-derived and common laboratory strains analyzed. In contrast, B-1a cells are present almost exclusively in the *Mus m. domesticus* subspecies. Bw cells present a unique phenotype distinct from that of B-1a, B-1b and B-2 cells. Transfer experiments establish that Bw cell differentiation is dependent on intrinsic cellular properties either from bone marrow or fetal liver origin. Bw cells stimulated by TLR ligands produce higher anti-PC Ab amounts than B-2 cells in vitro. Moreover, the lack of B-1a cells does not impair serum Ig levels even in 2 wild-derived strains whose MZ B cells show an unusual phenotype and a non classical structure of the splenic white pulp. Altogether, these data show that the B-1a population is not representative of the peritoneal B cell populations of the whole genus *Mus* and reveal a previously undescribed evolutionary conserved B cell population which may play a key role in early Ab responses.

## Materials and Methods

### Mice

C57BL/6 mice were purchased from Janvier. The following wild-derived mouse strains: BIK/g/Pas, MAI/Pas, MBT/Pas, PWK/Pas, SEG/Pas, STF/Pas, WLA/Pas, WMP/Pas and ZYD/Pas are maintained as inbred strains in the animal facility of Pasteur Institute (Paris, France). The 39 other wild-derived strains are maintained in outbred conditions in the laboratory of Dr. François Bonhomme (CNRS UMR5000 "Génome, Populations, Interaction" Université des Sciences et Techniques du Languedoc). They were used at 6 to 8 wk of age for FACS analysis, in vitro functional assays. Levels of immunoglobulins in unmanipulated animals were measured at 37 wk of age.

### Reagents

LPS from *Salmonella typhimurium* (Difco Laboratories) and re-purified by the classical phenol/water method (26). Phosphothioate-CpG (ODN 1826: TCCATGACGTTCTGACGTT) was synthesized by Invitrogen. Anti-CD40 mAb, a kind gift of Dr. Sedlick (Institut Curie, Paris, France) were used as a supernatant of FGK45 hybridoma cell line diluted 1:4. F(ab')<sub>2</sub> fragments of rabbit anti-mouse IgM Abs were prepared by pepsin digestion (27).

### Cells

Peritoneal cells were harvested by washing the peritoneal cavity from untreated animals with a total of 4 ml of RPMI 1640/3% FCS per individual. PBL were obtained by separation on a Ficoll gradient according to standard protocol.

### FACS analysis and cell sorting

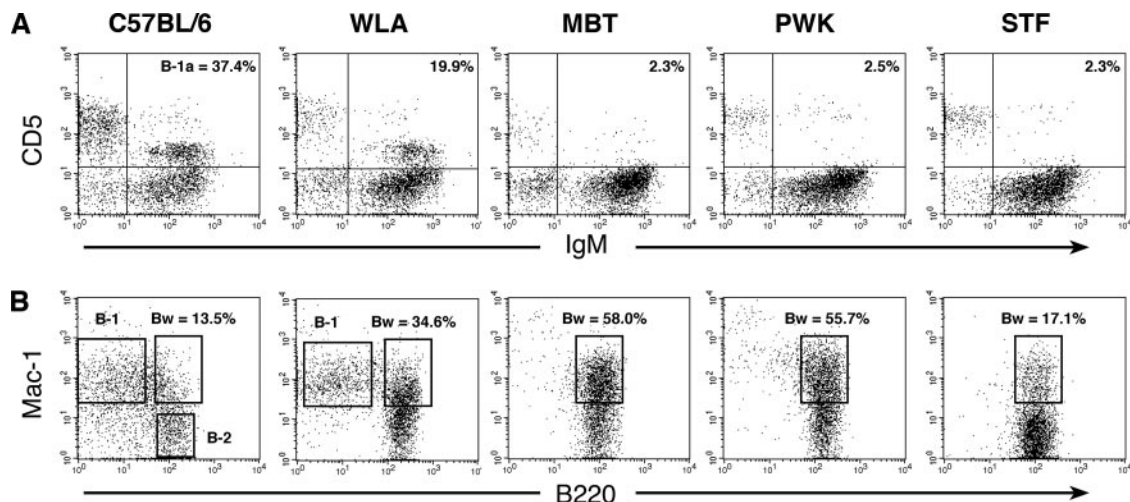
FITC or PE-labeled anti-IgM Abs were purchased from Jackson (Beckman Coulter) and PE-labeled anti-IgD from Southern Biotechnology. FITC-labeled anti-CD5, CD21, B220, IgM or PE-labeled anti-IgM, Mac-1, CD23, IgD or APC-labeled anti-B220 or biotinylated-labeled anti-CD1d, CD43 and CD9 Abs and unlabeled 2.4G2 used as Fc block (BD Bioscience Pharmingen), were used for FACS analysis. Lymphoid cells were gated on forward and side scatters, and dead cells were gated out using propidium iodide staining. FACS analyses were conducted using a FACSCalibur and the CellQuest 3.1 software from BD Biosciences. For cell sorting, peritoneal cells were usually harvested from 5 C57BL/6 and 10 to 15 wild derived strains. Forward and size scatter gated peritoneal cells stained by PE-labeled anti-Mac-1 and APC-labeled anti-B220 Abs were sorted using a MoFlo Cellsorter. The purity of sorted populations was  $\geq 97\%$ .

### Cultures for cytokine and Ig synthesis

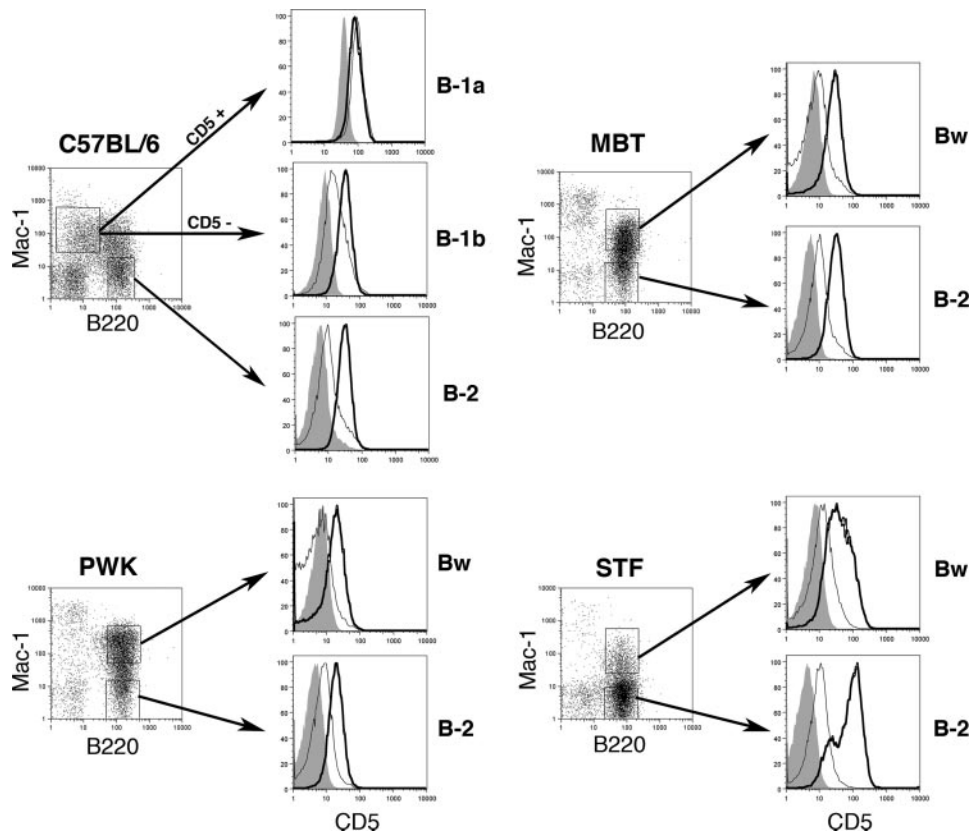
Sorted peritoneal cells were cultured at  $0.5 \times 10^6$ /ml for cytokine secretion or  $1 \times 10^6$ /ml for immunoglobulins/anti-PC Ab synthesis/FACS analysis of CD5 expression/in either 100  $\mu$ l of regular RP10 or 1.5 ml (autoantibody synthesis) in the presence of 10  $\mu$ g/ml LPS or 1  $\mu$ g/ml CpG or 10  $\mu$ g/ml F(ab')<sub>2</sub> fragment of rabbit anti-mouse IgM in 96- or 24-well culture plates. According to preliminary experiments, supernatants were collected after 2 days (cytokines/CpG), 3 days (cytokines/LPS and CD5 up-regulation/F(ab')<sub>2</sub> anti-IgM) or after 7 days for Ig or Ab synthesis in the presence of either LPS or CpG.

### Cytokine and Ig titers

The concentrations of IL-6 or IL-10, in the culture supernatants, were determined by ELISA using Duoset kits (R&D System).



**FIGURE 1.** FACS analysis of peritoneal B cells from laboratory mice (C57BL/6), *M. musculus domesticus* (WLA), *M. musculus musculus* (MBT, PWK), and *Mus spretus* (STF). *A*, Peritoneal cells stained with anti-CD5 and anti-IgM Abs in the presence of Fc block were gated on lymphocytes according to FSC/SSC criteria and dead cells excluded by propidium iodide gating. Numbers in the upper right quadrants indicate the proportion of B-1a cells among the whole B cell population. *B*, Peritoneal Bw cells labeled with anti-Mac-1 and anti-B220 Abs were gated on IgM<sup>+</sup> cells. Percentages indicated for Bw cells are among total B cells. These plots are representative of several similar experiments. Similar data were obtained in the presence or in the absence of Fc block.



**FIGURE 2.** Expression of CD5 on activated peritoneal B cell subpopulations. B-1a cells were sorted on the basis of B220<sup>low</sup>Mac-1<sup>+</sup>CD5<sup>+</sup> staining, B-1b cells as B220<sup>low</sup>Mac-1<sup>+</sup>CD5<sup>-</sup> and B-2 cells as B220<sup>high</sup>Mac-1<sup>-</sup> from C57BL/6 mice, Bw cells were sorted on the basis of B220<sup>high</sup>Mac-1<sup>+</sup> staining and B-2 cells as B220<sup>high</sup>Mac-1<sup>-</sup> from MBT, PWK and STF according to the gate indicated on each dot plots. Membrane expression of CD5 was analyzed by FACS before (gray histograms) or following a 3 day culture at  $1 \times 10^6$  cells/ml in the absence (thin lines) or the presence of 10  $\mu\text{g/ml}$  F(ab')<sub>2</sub> anti-IgM Ab and 25% anti-CD40 Ab supernatants (thick lines).

IgM and IgG titers in preimmun sera were determined by ELISA using the Southern Biotechnology Associates kit (cat. no. 5300.05), according to manufacturer's recommendations. Titers were calculated on the basis of reference absorbance values of standard curve of IgM or IgG immunoglobulins (Jackson, Beckman Coulter).

Anti-PC Ab titers were determined as follows: phosphorylcholine (Sigma-Aldrich) coupled to BSA (ICN Pharmaceuticals) (PC-BSA) as previously described (28) was coated at 10  $\mu\text{g/ml}$  at room temperature for 2 h. After two washes with PBS/0.5% tween and a 30 min saturation with PBS/tween/1% BSA, serial dilutions of culture supernatants or known concentrations of HOPC8 myeloma protein (IgA $\kappa$  anti-PC) were allowed to incubate overnight at 4°C. After 3 washes, bound Abs were then amplified using peroxidase labeled anti-H+L anti-IgG (Southern Biotechnology Associates) and revealed by *o*-phenylenediamine (OPD) (Sigma-Aldrich) in the presence of H<sub>2</sub>O<sub>2</sub> according to manufacturer's recommendations.

Autoantibody titers were determined according to same protocol after coating of purified tubulin, actin and DNA (a kind gift of Dr. Thérèse Ternynk) used at 10  $\mu\text{g/ml}$ . Data are expressed as ELISA units (EU).

#### Proliferation assays

Peritoneal cells labeled as indicated in the figure legends were sorted using a MoFlo cytometer and plated in RPMI 1640-glutamax (Invitrogen) supplemented with 10% FCS, penicillin, streptomycin, 2 mM pyruvate, 50 M 2-ME (regular RP10) at  $2 \times 10^5$  cells/ml in 96-well culture plates (TPP, ATGC) at 37°C in 7% CO<sub>2</sub> in the presence or the absence of 1  $\mu\text{M}$  CpG or of 10  $\mu\text{M}$  LPS for 48 and 72 h, respectively. Cultures were pulsed with 1  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine (1 Ci = 37 GBQ) 6 h before the end of the culture. Results are expressed as  $\delta$  cpm (mean value of triplicate minus background).

#### Cultures and ELISA for the frequencies of anti-tubulin, anti-actin and anti-DNA-specific B cells

Peritoneal Bw, B-1, or B-2 cells were sorted according to their Mac-1/B220 expression from 5 C57BL/6, 12 WLA, 13 MBT, 12 SEG, 10 PWK,

12 STF, and 10 WMP. Cells were cultured at  $5 \times 10^5$ /ml in 24-well culture plate, with both CpG (1  $\mu\text{g/ml}$ ) and anti-CD40 (1/4). Supernatants were harvested after 7 days culture and tested by ELISA for the presence of anti-tubulin, anti-actin, and anti-DNA. Tubulin, actin, DNA (a kind gift of Dr. T. Ternynk) were coated at 5  $\mu\text{g/ml}$  at room temperature for 2 h. ELISA was made as described above for the anti-PC Abs. The autoantibody specificities were then amplified using peroxidase-labeled anti-H+L anti-IgG (Southern Biotechnology Associates) and revealed by *o*-phenylenediamine (OPD) (Sigma-Aldrich) in the presence of H<sub>2</sub>O<sub>2</sub> according to manufacturer's recommendations. Data are expressed as explained in Table III.

#### Frequencies of BrMRBC-specific B cells

Erythrocytes were treated with bromelain as previously described (13). Briefly, 1 vol of C57BL/6J erythrocyte pellet was resuspended with 1 vol of bromelain (Sigma, B-2252) at 10 mg/ml in PBS for 60 min at 37°C. After incubation period, pellet was washed 3 times with PBS and resuspended at 2% in RPMI 1640/3% FCS. Peritoneal and spleen cells ( $5 \times 10^6$  cells/ml) were maintained 2 h at 4°C with the same volume of BrMRBC (2%) in 96 flat-bottom microplates (TPP, ATGC). Thereafter, cells were gently resuspended and counted in double-blind.

#### Fetal liver transfers

Bone marrow cells ( $7 \times 10^6$ ) depleted in CD4<sup>+</sup> and CD8<sup>+</sup> T and CD19<sup>+</sup> B cell by magnetic cell sorting (Miltenyi Biotec) or  $7 \times 10^6$  fetal liver cells from PWK (Ly5.1) or from Ly5.1 congenic C57BL/6 harvested at day 14 of gestation, were injected i.v. in 6 Gy irradiated Rag2  $\gamma\text{c}$ -deficient C57BL/6 (Ly5.2) mice. FACS analyses were conducted on peritoneal cells 6 wk after transfer.

## Results

### A novel peritoneal B cell population in the genus *Mus*

Contrary to what is observed in laboratory mouse strains, CD5<sup>+</sup> B cells are not representative of peritoneal B cell populations found in various species (2). For this reason, we have revisited the phenotype of peritoneal cells among wild-derived strains belonging to the genus *Mus*.

FACS profiles of peritoneal cavity cell populations (Fig. 1A) and of peritoneal IgM<sup>+</sup> B cells (Fig. 1B) depict the phenotypes observed in C57BL/6 laboratory strains and in four representative wild-derived (WLA, MBT, PWK and STF) strains among the nine inbred wild-derived mouse strains analyzed. The B-1a population (CD5<sup>+</sup>Mac-1<sup>+</sup>B220<sup>low</sup>IgM<sup>high</sup>) is easily observed in C57BL/6 control mice and in WLA (*M. musculus domesticus*). This population is largely absent from the peritoneal cavity of MBT, PWK and STF, the two first belonging to *M. musculus musculus* and the last one to *Mus spretus* (Fig. 1A). It should be noted that in all these strains, T cells display high levels of the CD5 Ag (Fig. 1A, upper right quadrant), ruling out that a putative polymorphism at the CD5 level could prevent binding of the anti-CD5 mAb.

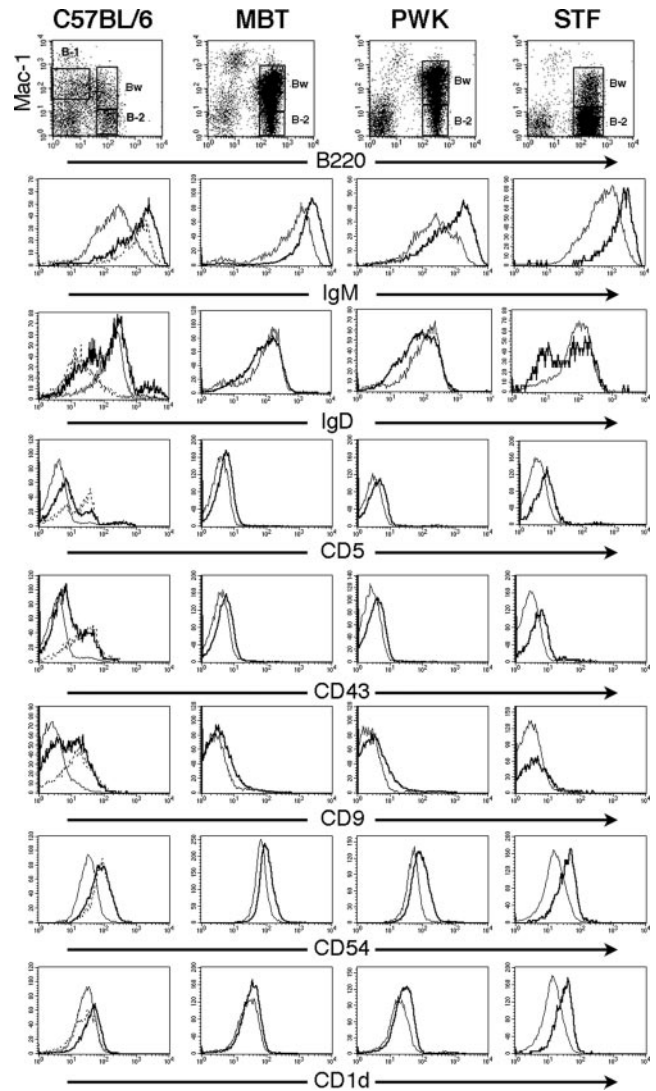
Analysis of Mac-1/B220 expression on gated IgM<sup>+</sup> B cells confirms the absence of the B-1a subset in MBT, PWK, and STF and shows that the B-1b (CD5<sup>-</sup>Mac-1<sup>+</sup>B220<sup>low</sup>IgM<sup>high</sup>) subset is also missing in these strains. Moreover, FACS analysis reveals the presence of a previously undescribed population of Mac-1<sup>+</sup>IgM<sup>+</sup> B cells in the peritoneal cavity of all mice. This novel population expresses high density of B220 in contrast to the classical B-1 cells, which are B220<sup>low</sup> as observed in C57BL/6 control mice and in WLA (Fig. 1B).

Due to the large frequency of this novel B cell population (13 to 58% of all B cells) in the peritoneal cavity and its discovery in wild-derived mouse strains, we propose to name this CD5<sup>-</sup>Mac-1<sup>+</sup>B220<sup>high</sup>IgM<sup>+</sup> population: the Bw population, “w” referring to wild-derived mice. Nevertheless, careful analysis of FACS profiles revealed that the Bw population is also found in common laboratory strains, such as in C57BL/6 (Fig. 1B).

To investigate directly the absence of constitutive expression of the CD5 glycoprotein at the membrane of B cells in wild-derived mouse strains, B-1a, B-1b and B-2 cells from C57BL/6 or Bw and B-2 cells from MBT, PWK and STF were purified by cell sorting, according to the gates shown in (dot plots Fig. 2). Purified cells (>97% of purity in all cases) were cultured for 2 days in the presence of F(ab')<sub>2</sub> fragments of rabbit anti-mouse IgM Abs and anti-CD40 mAbs and then analyzed for CD5 expression. FACS analysis indicate that the level of CD5 in B-1a cells, which constitutively express CD5, is not increased in these culture conditions, as compared with cells cultured alone. In contrast, both B-1b and B-2 cells up-regulate CD5 when stimulated through the BCR (Fig. 2). Similarly, Bw and B-2 cells from MBT, PWK and STF are able to up-regulate CD5.

Altogether, these data show that, upon activation, B-1b and B-2 from C57BL/6 and Bw and B-2 cells from wild-derived mouse strains are able to express membrane CD5 Ag, demonstrating that the CD5 expression pathway is not impaired in Bw and B-2 cells in mice which lack CD5<sup>+</sup> B cell subset.

To further characterize the phenotype of Bw cells, FACS analyses of peritoneal lymphoid cells from C57BL/6, MBT, PWK, and STF were performed. The phenotype of peritoneal Bw cells from 3 different wild-derived mice strains is: Mac-1<sup>+</sup>B220<sup>high</sup>IgM<sup>high</sup>IgD<sup>high</sup>CD19<sup>+</sup>CD5<sup>-</sup>CD43<sup>-</sup>CD9<sup>-</sup>CD54<sup>high</sup>CD1d<sup>+</sup> (Fig. 3). Thus, they are Mac-1<sup>+</sup>IgM<sup>high</sup>CD54<sup>high</sup> as B-1 cells and CD5<sup>-</sup>B220<sup>high</sup>IgD<sup>high</sup>CD43<sup>-</sup>CD9<sup>-</sup> as B-2 cells. It should be noted that, based on the level of Mac-1 expression, the Bw cell population appears to be heterogeneous.



**FIGURE 3.** Phenotype of Bw cells. Labeled peritoneal cells were gated on lymphocytes according to FSC/SSC criteria and dead cells exclusion by propidium iodide. Histograms measure the expression of IgM, IgD, CD5, CD43, CD9, CD54, CD1d molecules by cells gated on Mac-1<sup>+</sup>B220<sup>high</sup> (Bw cells: thick line), Mac-1<sup>+</sup>B220<sup>low</sup> (B-1 cells: dotted line) and Mac-1<sup>-</sup>B220<sup>low</sup> phenotype (B-2 cells: fine line). This experiment is representative of several similar experiments. Bw profiles must be compared with that of peritoneal B cells from C57BL/6. A positive staining with anti-CD5, CD43 and CD9 Abs of some C57BL/6 peritoneal B cells can be observed in cells gated on the Bw population which most probably reflects the overlap in this strain of B-1 and Bw cells.

These data indicate that the Bw share some phenotypic features with other B cell subsets but as a whole display an original phenotype which suggests that the Bw subset is distinct from that of both B-1a, B-1b and B-2 cell subsets.

### Peritoneal B cells phenotype of 48 wild-derived mice and 7 laboratory mice

In addition to the 9 inbred wild-derived strains analyzed, we studied 39 outbred wild-derived strains, whose progenitors were captured in different places of Europe and Asia and belong to 13 species or subspecies of the genus *Mus* (Fig. 4A). The results clearly show that the presence of CD5<sup>+</sup> B cells is mostly restricted to strains (13/16) belonging to the *Mus. m. domesticus* subspecies.

Fig. 4A also shows that the 48 strains analyzed have the Bw population. Moreover, in addition to B-2, different combinations of

**A**

Species/ subspecies	n	B cell population			
		B-1 cells Mac-1+ B220 low	Bw cells Mac-1+ B220 high	B-1a cells IgM high CD5+	
<i>M. musculus domesticus</i>	BIK/g*	>10			
	BZO	2			
	DCA	2			
	DGP	2			
	DDO	2			
	DEB	2			
	DGA	2			
	DIK	2			
	DJO	4			
	DMZ	2			
	DOT	4			
	22MO	4			
	WLA*	>10			
	WMP*	>10			
	<i>M. musculus musculus</i>	MAI*	>10		
		MAM	2		
		MBK	2		
		MBS	2		
		MBT*	>10		
		MDH	4		
		MGA	2		
		MPB	2		
PWK*		>10			
<i>M. musculus castaneus</i>		CIM	4		
		CKN	2		
	CKS	2			
	CTA	2			
	CTP	2			
<i>M. musculus molossinus</i>	MOL	2			
	BID	2			
<i>M. musculus spp</i>	DHA	3			
	KAK	2			
	MAC	2			
	MPR	4			
	TEH	2			
	DKN	2			
<i>M. musculus dom-cast gentilulus</i>	MDG	2			
<i>Mus cypriacus</i>	YCA	2			
<i>Mus spicilegus</i>	ZRU	2			
	ZYD*	>10			
<i>Mus macedonicus</i>	XBS	2			
<i>Mus spretus</i>	SEB	2			
	SEG*	>10			
	SFM	2			
	SMZ	2			
	STF*	>10			
<i>Mus famulus</i>	FAM	2			
<i>Mus caroli</i>	KTK	2			

**B**

Laboratory strains	n	B-1 cells Mac-1+ B220 low	Bw cells Mac-1+ B220 high	B-1a cells IgM high CD5+
129/Sv	6			
BALB/c	>10			
C3H/HeJ	4			
C57BL/6J	>10			
CBA	4			
DBA/1	5			
DBA/2	5			
NOD	3			
(NZBxNZW)	4			
SJL	>10			
SWISS	>10			

**FIGURE 4.** Peritoneal B cell phenotypes of 48 wild-derived mice and 7 laboratory strains. *A*, B cell population from the peritoneal cavity of 48 wild-derived and (*B*) 7 laboratory mouse strains were analyzed by flow cytometry. The latter are representative of each group of the Petkov's mouse family tree (29). Motifs denote the percentage of a given B cell population among total peritoneal B cells as follows: frequencies greater than 10% (dark gray), between 5 and 10% (light gray), and lesser than 5% (white). Number of mice tested for each strain is indicated in column *n*. Boxes for a given population are divided to show individual mice when different phenotypes were found. The 9 strains scored with an asterisk, namely BIK/g, MAI, MBT, PWK, SEG, STF, WLA, WMP, ZYD are maintained in inbred conditions.

cell subsets can be observed: Bw (Mac-1<sup>+</sup>/B220<sup>high</sup>), B-1a (CD5<sup>+</sup>IgM<sup>high</sup>) and B-1b (Mac-1<sup>+</sup>/B220<sup>low</sup>) such as, for instance, in WLA (*Mus. m. domesticus*), Bw and B-1b such as in MDH

(*Mus. m. musculus*) or Bw alone such as in MBT (*Mus. m. musculus*). Data indicate also that B-1b cells are found in variable proportions in mice from a given strain probably due to their out-bred status.

The fact that laboratory mice are all originated from the crossing in unequal proportions of *M. musculus domesticus*, *M. musculus musculus* and *M. musculus castaneus* which all have peritoneal Bw cells, prompted us to search for this population in 7 laboratory mice genetically distant from each other as defined in the Petkov's mouse family tree (29). FACS analysis revealed that they all have the novel Bw cell population in addition to the canonical CD5<sup>+</sup> B-1 population (Fig. 4*B*).

Altogether these data provide evidence for the existence of a significant frequency of Bw cells in all laboratory and wild-derived strains studied and clearly show that in contrast to Bw cells, B-1a cells are not representative of the whole peritoneal B cell composition.

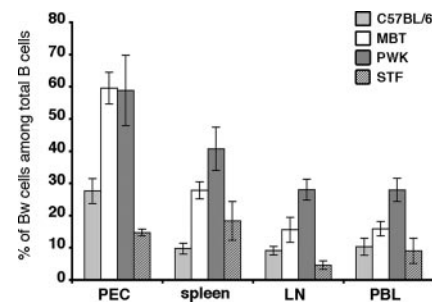
#### Frequency of Bw cells in different lymphoid organs

To analyze the anatomical distribution of Bw cells based on the CD19<sup>+</sup>Mac-1<sup>+</sup>B220<sup>high</sup> phenotype, we determined their percentage among total B cells in peritoneal cavity, spleen, lymph nodes and PBL of C57BL/6, MBT, PWK, and STF (Fig. 5). In MBT and PWK, the Bw cells among total B cells are found at higher frequency in the peritoneal cavity (60%) and at a lower frequency in the spleen (28–41%), the lymph nodes and PBL (10–28%). In STF, Bw cells were found in roughly similar frequencies in peritoneal cavity (15%) and spleen (18%) and at much lower frequency in the lymph nodes (5%) and PBL (9%). However, Table I shows that the absolute number of Bw cells is much higher in the spleen than in peritoneal cavity and any other organs. It is noteworthy that the presence of Bw cells in C57BL/6 lymphoid organs was confirmed by the absence of positive signals with control isotypes labeled with the same fluorochromes (data not shown). In addition, the data show that B cell frequencies are almost conserved while the absolute number of total splenic B cells varies among the strains which can be explained by their different genetic background.

Altogether, these data indicate that Bw cells are not restricted to the peritoneal cavity. Moreover, their presence in PBL of all strains suggests that they are able to recirculate.

#### Splenic B cells phenotype

We also investigated the relative distribution of FO B cells (B220<sup>high</sup>Mac-1<sup>-</sup>IgM<sup>low</sup>IgD<sup>high</sup>CD21<sup>low</sup>CD23<sup>high</sup>CD1d<sup>low/neg</sup>) and



**FIGURE 5.** Percentage of Bw cells in different lymphoid organs. Bw cells were quantified by flow cytometry using Mac-1/B220 staining gated on CD19 positive cells in peritoneal cavity, spleen, lymph nodes and PBL from MBT, PWK, STF and C57BL/6 as a control. The results are expressed as percentage of Bw cells among the total B cells in each organ. These data are representative of several experiments performed with adult mice (15–45 wk), with or without Fc block and are presented as mean value of 4 mice per strain ± SD.

Table I. Absolute number of nucleated cells and B cell subpopulations in various organs<sup>a</sup>

		PEC	Spleen	LN	PBL
C57BL/6	Total nucleated cells	4.07 ± 0.76	109.55 ± 19.89	24.93 ± 4.86	2.32 ± 0.64
	Total B	1.52 ± 0.47	52.54 ± 13.17	8.77 ± 3.63	0.25 ± 0.06
	B-1	0.49 ± 0.10	0.13 ± 0.05	<0.01	<0.01
	Bw	<b>0.42 ± 0.17</b>	<b>4.98 ± 0.81</b>	<b>0.77 ± 0.23</b>	<b>0.026 ± 0.005</b>
	B-2	0.60 ± 0.24	47.43 ± 12.43	7.99 ± 3.42	0.216 ± 0.051
MBT	Total nucleated cells	1.94 ± 0.25	82.47 ± 41.27	5.55 ± 1.52	2.24 ± 0.82
	Total B	0.44 ± 0.09	30.79 ± 6.57	1.26 ± 0.29	0.20 ± 0.09
	B-1	0.01 ± 0.01	0.06 ± 0.02	<0.01	<0.01
	Bw	<b>0.27 ± 0.07</b>	<b>8.57 ± 1.89</b>	<b>0.20 ± 0.07</b>	<b>0.033 ± 0.011</b>
	B-2	0.16 ± 0.03	22.16 ± 4.86	1.06 ± 0.25	0.166 ± 0.076
PWK	Total nucleated cells	4.77 ± 2.23	66.34 ± 22.22	10.33 ± 3.82	3.72 ± 1.19
	Total B	1.03 ± 0.39	29.73 ± 11.21	2.67 ± 1.19	0.24 ± 0.10
	B-1	0.02 ± 0.01	0.42 ± 0.43	<0.01	<0.01
	Bw	<b>0.59 ± 0.20</b>	<b>12.46 ± 6.05</b>	<b>0.72 ± 0.27</b>	<b>0.078 ± 0.030</b>
	B-2	0.42 ± 0.28	17.17 ± 5.58	1.94 ± 0.93	0.159 ± 0.069
STF	Total nucleated cells	1.81 ± 0.58	43.63 ± 15.99	10.05 ± 4.13	1.73 ± 0.58
	Total B	0.71 ± 0.13	17.26 ± 5.39	2.46 ± 1.31	0.30 ± 0.08
	B-1	0.01 ± 0.01	0.07 ± 0.07	<0.01	<0.01
	Bw	<b>0.10 ± 0.01</b>	<b>3.01 ± 1.08</b>	<b>0.10 ± 0.03</b>	<b>0.015 ± 0.004</b>
	B-2	0.60 ± 0.12	14.18 ± 5.09	2.35 ± 1.29	0.279 ± 0.072

<sup>a</sup> Nucleated cells were counted in the presence of trypan blue to exclude dead cells. Total B (IgM<sup>+</sup>B220<sup>+</sup>CD19<sup>+</sup>), B-1 (Mac-1<sup>+</sup>IgM<sup>hi</sup>B220<sup>lo</sup>), Bw (Mac-1<sup>+</sup>IgM<sup>hi</sup>B220<sup>hi</sup>), B-2 (Mac-1<sup>+</sup>IgM<sup>lo</sup>B220<sup>hi</sup>) were assessed by FACS in the presence of propidium iodide as described in the *Materials and Methods* section. Data are expressed as mean value of the number of cells × 10<sup>-6</sup>/organ from 4 individual mice/strain ± SE except in PBL isolated by Ficoll where numbers are expressed for 1 ml of blood. Data in bold highlight the number of Bw cells in each organs

MZ B cells (B220<sup>high</sup>Mac-1<sup>-</sup>IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>high</sup>CD23<sup>low</sup>CD1d<sup>high</sup>) in the 9 inbred wild-derived strains.

Fig. 6 shows the phenotypic characterization of the splenic B cell populations of C57BL/6, MBT, PWK and STF stained with Mac-1/B220, IgM/IgD, CD21/CD23 or CD1d/CD21 mAbs. The upper quadrants (gated on CD19, 1st row) show typical stainings of the Bw subset (Mac-1<sup>+</sup>B220<sup>high</sup>) and confirm data reported on

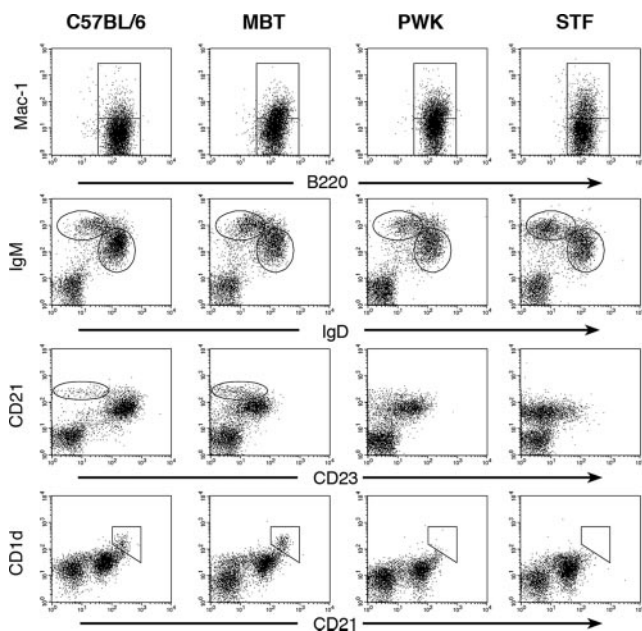
Fig. 6. The FACS profiles shown on the 2nd row reveals that the IgM/IgD profiles are similar between all strains. Unexpectedly, we found that PWK and STF splenic B cells have an unusual phenotype characterized by a slight decrease in CD21 expression, down-regulation of CD23, absence of CD1d<sup>high</sup> cells and of CD9<sup>+</sup> cells (Fig. 6, 3rd and 4th row and data not shown).

*In vitro functional characteristics of Bw cells in wild-derived mouse strains*

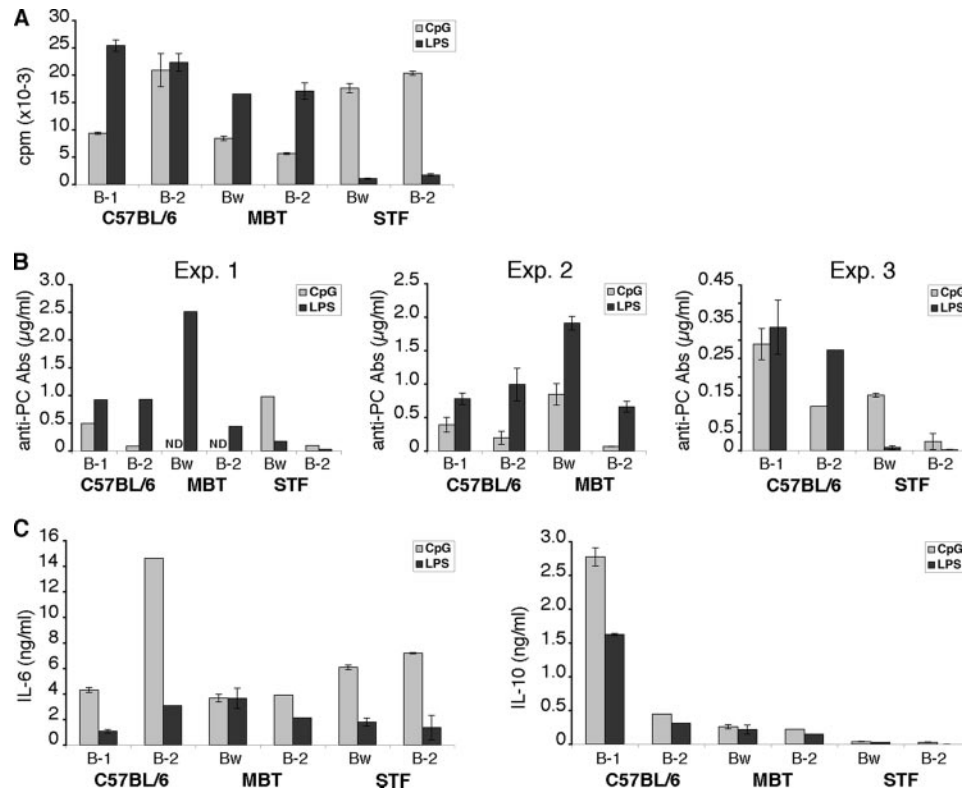
The heterogeneity of B cell phenotype in spleen led us to focus on the characterization of peritoneal Bw cells. It has been shown that MZ B cells proliferate more readily than B-2 cells to TI Ags (3). To investigate the functional properties of Bw cells, we sorted B-1, Bw and B-2 cells from a pool of 4 C57BL/6, 12 MBT and 15 STF per experiment, according to the gates shown in Fig. 2. The PWK strain was not included in this study due to its very low response to both TLR ligands in the absence of further costimulations (data not shown).

Data show that, Bw cells do not proliferate more than B-2 cells, in response to CpG or LPS (Fig. 7A). Note also that in the presence of F(ab')<sub>2</sub> anti-mouse IgMs, Bw cells from MBT mice show an intermediate profile of proliferation when compared with that of B-1 and B-2 cells from C57BL/6. In contrast, B-2 cells from both MBT and C57BL/6 show similar profiles in both <sup>3</sup>TH incorporation and CFSE experiments (data not shown). Bw cells from MBT and STF produced more anti-PC Abs than B-2 cells after either CpG or LPS stimulation (Fig. 7B). Similarly, B-1 cells from C57BL/6 produced more anti-PC Abs than B-2 cell counterparts in the presence of CpG but similar levels of anti-PC LPS stimulation.

The ability of Bw cells to secrete IL-6 and IL-10, two major cytokines produced by B cells, in response to CpG and LPS is shown in Fig. 7C. Bw and B-2 cells from MBT and STF synthesize roughly similar levels of IL-6 upon CpG or LPS stimulation. In contrast, B-2 cells from C57BL/6 are much better producers of IL-6 than B-1 cells (right panel). In similar culture conditions, B-1 cells synthesize high levels of IL-10 in comparison with their B-2 counterparts and with both Bw and B-2 cells from MBT and STF strains. It should also be noted that Bw cells do not constitutively



**FIGURE 6.** Splenic B cell phenotype. FACS analysis of splenic B cells from C57BL/6, MBT, PWK (*M. m. musculus*) and STF (*Mus spretus*) stained with anti-Mac-1, anti-B220, anti-IgM, anti-IgD, anti-CD21, anti-CD23 and anti-CD1d Abs. Gates indicate: Bw cells (upper gate 1<sup>st</sup> row), B-2 cells (lower gate 1<sup>st</sup> row); IgM<sup>high</sup>IgD<sup>low</sup> (2<sup>nd</sup> row left), IgM<sup>low</sup>IgD<sup>high</sup> (2<sup>nd</sup> row right); MZB cells (CD21<sup>high</sup>CD23<sup>low</sup>, 3<sup>rd</sup> row) which are detected in C57BL/6 and MBT but not in both PWK and STF; (CD21<sup>low</sup>CD23<sup>high</sup>, 4<sup>th</sup> row) which are detected in C57BL/6 and MBT but not in both PWK and STF.



**FIGURE 7.** In vitro responses of sorted peritoneal B cells upon CpG and LPS stimulations. *A*, Proliferative responses of sorted peritoneal B cells were studied at the peaks of <sup>3</sup>H-thymidine incorporation i.e., after 48 h of CpG or 72 h of LPS stimulation, according to preliminary experiments. Data are expressed as mean value of triplicate  $\pm$  SD and are from one experiment representative of several similar for C57BL/6 and two for each of the wild-derived strains. <sup>3</sup>H-thymidine incorporations were  $<400$  cpm in unstimulated cultures. *B*, In vitro induction of anti-PC Abs by CpG or LPS from peritoneal sorted B cells. Supernatants were harvested 7 days after stimulation and anti-PC Ab levels were determined by ELISA. Data of 3 experiments are presented, expressed as ng/ml HOPC8 anti-PC Ab equivalent and presented as mean value of duplicate cultures  $\pm$  SD. The concentrations of anti-PC Abs in unstimulated cultures were  $<2$  ng/ml. *C*, In vitro induction of IL-6 and IL-10 by peritoneal sorted B cells following a 48 h culture with CpG or a 72 h culture with LPS. Cytokine levels were determined in the culture supernatants by ELISA. Data are representative of 2 similar experiments and presented as mean value of duplicate cultures  $\pm$  SD. IL-6 levels in unstimulated cultures were  $<0.7$  ng/ml and  $<0.5$  ng/ml at 48 and 72 h of culture, respectively. IL-10 levels in unstimulated cultures were  $<0.02$  ng/ml at either 48 or 72 h except in B-1 cultures where they reached 0.15 ng/ml.

secrete IL-10, in contrast to B-1 cells, which have already been described to be the major source of IL-10 (30).

Altogether, these data show that Bw cells are better producer of anti-PC Abs than B-2 cells, similarly to B-1 upon CpG stimulation, and that they behave as their B-2 counterparts in terms of cytokine secretion or proliferative response. These results confirm at the functional level that the Bw subset is distinct from the B-1 and B-2 cell populations.

#### Autoantibody enrichment in peritoneal Bw cells

A feature of the B-1a Ab repertoire in laboratory strains is the high frequency Abs recognizing autoantigens and among which a phosphatidylcholine group (PtC) unmasked by treatment of mouse RBC with bromelain (BrMRBC). This specificity is reduced in laboratory mice lacking B-1a cells (31). To seek for a possible redundancy between B-1a and Bw Ab repertoire, we evaluated the proportion of BrMRBC-specific peritoneal B cells in wild-derived mice. Data reported on Table II are representative of three experiments. We found that only B cells from mice having the peritoneal B-1a population (C57BL/6 and WLA) were able to form rosettes with BrMRBC at high frequency ( $>15\%$ ). However, peritoneal cells from MBT and SEG, which do not have B-1a cells, could reproducibly form rosettes with BrMRBC although at a lower frequency (2–3%). Very low frequency of morulla-like rosettes were reproducibly found with peritoneal B cells from PWK, STF, and

WMP. Similar results were obtained with BrMRBC originated from each of the wild-derived inbred strains (data not shown). Altogether, these data show that the lack of B-1a cells reduces considerably but does not abrogate the BrMRBC rosette-forming cells, suggesting that the Bw cell subset contain a low but significant frequency of BrMRBC Ab specificity.

We next asked whether the Ab repertoire of Bw cells was enriched against 3 other autoantigen specificities. To this aim, we stimulated Bw or B-2 cells from several wild-derived mice and B-1 or B-2 cells from C57BL/6 as a control, with CpG and anti-CD40, to bypass the heterogeneity of responses of the different wild-derived mice against TLR ligands (data not shown) and screened the culture supernatants for the presence of anti-tubulin, anti-actin and anti-DNA autoantibodies (Table III). The results expressed in ELISA units (EU) clearly show that Bw cells are enriched in one, two or these three specificities depending on the wild strain considered. This is still true when the Bw/B-2 ratio for a given specificity is compared with that of total Igs. It is noteworthy that in several cases, autoantibodies are not detectable in the supernatant of B-2 cell cultures (EU  $<0.10$ ) although the same culture supernatants contain high titer of total Igs.

Altogether, these data show clearly that peritoneal Bw cells from distinct strains are variously enriched in autoantibody specificities.



Table II. Frequencies of BrMRBC-specific B cells in wild mice<sup>a</sup>

	Peritoneal Cells	Splenocytes
C57BL/6	1757 ± 255	20 ± 20
WLA	2518 ± 258	33 ± 23
MBT	<b>247 ± 47</b>	24 ± 21
SEG	<b>286 ± 142</b>	21 ± 22
PWK	79 ± 29	11 ± 7
STF	87 ± 25	28 ± 30
WMP	47 ± 43	8 ± 9

<sup>a</sup> Data from one out of four experiments, counted in double-blind, are reported and expressed as number of rosette forming cells (RFC) per 10<sup>4</sup> B cells. BrMRBC where from C57BL/6 origin. Similar data were found with autologous BrMRBC, however no rosettes could be detected with PBS treated MRBC (data not shown). B cell frequency in each suspension was determined by FACS analysis.

### Serum Ig titers

We then addressed the question of whether the absence of the classical B-1 population, together with the unusual splenic B cell phenotype (Fig. 6) would have an impact on the natural Ig levels in PWK and STF. Indeed, it has been described that B-1 and MZ B cells contribute substantially to serum IgM level in mice and human, respectively (8, 9, 32, 33). IgM and total Ig concentrations were measured in the serum of adult mice. We found that both serum IgM (Fig. 8A) and total Igs (Fig. 8B) concentrations vary from one strain to another. However, SEG, PWK, STF, and WMP, which have no CD5<sup>+</sup> B-1a cells, synthesize similar levels of IgMs than WLA which have this population. Only MBT show lower concentration of IgMs while they present a classical splenic structure and the canonical phenotype of splenic B cells (Fig. 6).

These data indicate that there is no direct correlation between the presence of B-1a cells, canonical splenic B cell phenotype and serum IgM levels. Altogether, these results led us to consider that the Bw cells can be implicated as B-1a and MZ B cells in the production of natural IgMs.

### Intrinsic capability of wild progenitors to differentiate in Bw cells

To determine whether Bw cell differentiation is under autonomous cell control, we transferred bone marrow (BM) or fetal liver (FL) precursors from PWK, expressing the Ly5.1 allotypic marker, and from Ly5.1 congenic C57BL/6 mice (B6-Ly5.1), as a control, into

irradiated Rag2  $\gamma$ c deficient mice (Ly5.2) and analyzed the peritoneal B cell populations 6 wk after transfer. The results show that similar frequencies of Bw cells were generated after reconstitution of Rag2  $\gamma$ c deficient mice with either adult BM or FL from B6-Ly5.1 or PWK, which in both cases reach a percentage of Bw cells close to their adult controls (Fig. 9A). Neither BM or fetal progenitors from PWK did give rise to B-1a cells (Fig. 9B). Fetal progenitors from B6-Ly5.1 led to the production of a significant higher level of B-1a cells than bone marrow progenitors, as already described and confirmed by the identification of the B-1 progenitor by Montecino et al. (17). As expected, the B-2 population reached similar proportions after transfer of either type of precursors (Fig. 9C).

Altogether, these data per se do not exclude peritoneal environmental influences but demonstrate that the ability to generate a Bw compartment is an intrinsic property of progenitors from FL or BM origin in wild mice. Moreover, these data suggest that the Bw subset can be originated from fetal liver progenitors during the first step of the development and from BM origin thereafter and during the adult life.

### Discussion

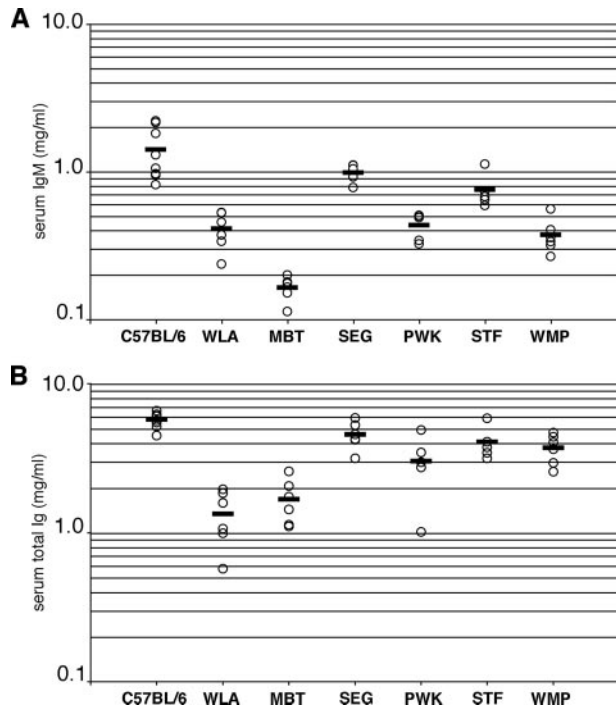
B-1a CD5<sup>+</sup> B cells have never been found in specific anatomic sites in vertebrates except in mice (2, 33). Therefore, to ascertain the singularity of the presence of CD5<sup>+</sup> B cells in laboratory mice, we have revisited the peritoneal B cell populations in 9 inbred and 39 outbred wild-derived mouse strains belonging to several species and subspecies. We found that CD5<sup>+</sup> B cells are not fully representative of the peritoneal B cell populations of the whole genus *Mus* and describe a novel Mac-1<sup>+</sup> B cell population represented in all the genus *Mus* which can both respond to TLR ligands and produce autoantibodies.

Our results show that CD5<sup>+</sup> peritoneal B cells are restricted to mice belonging to the *M. musculus domesticus* subspecies. Considering the fact that laboratory strains are derived from the crossing of a limited number of ancestors belonging to *Mus m. domesticus*, *Mus m. musculus* and *Mus m. castaneus* in unequal proportions (18), our findings strongly suggest that B-1a cells present in the vast majority of laboratory mice were inherited from the *M. musculus domesticus* subspecies. It is noteworthy that the absence of CD5<sup>+</sup> B cells does not reflect a polymorphism at the

Table III. Autoantibody enrichment in peritoneal B-1 or Bw cells compared to peritoneal B-2 cells<sup>a</sup>

		Titer				B-1 or Bw / B-2 ratio			
		Tubulin	Actin	DNA	Total Ig	Tubulin	Actin	DNA	Total Ig
C57BL/6	B-1	35.89	37.40	13.96	9646	1.38	1.39	1.43	1.11
	B-2	26.05	26.88	9.74	8625				
WLA	Bw	9.71	10.64	16.75	8282	1.68	1.36	<b>4.69</b>	1.06
	B-2	5.78	7.83	3.57	7802				
MBT	Bw	32.15	35.87	12.95	9446	<b>2.76</b>	1.22	1.08	1.07
	B-2	11.64	29.30	11.93	8834				
SEG	Bw	26.54	32.03	25.09	9846	<b>&gt;26.00</b>	<b>3.87</b>	<b>&gt;25.00</b>	0.76
	B-2	<0.10	8.27	<0.10	12904				
PWK	Bw	24.95	27.52	27.10	9685	<b>2.50</b>	1.83	<b>&gt;27.00</b>	0.96
	B-2	10.00	15.04	<0.10	10107				
STF	Bw	28.83	30.53	6.70	12100	<b>33.51</b>	<b>6.46</b>	<b>&gt;6.00</b>	1.52
	B-2	0.86	4.73	<0.10	7966				
WMP	Bw	49.17	56.04	13.45	10956	<b>4.44</b>	2.11	1.59	1.47
	B-2	11.07	26.53	8.46	7429				

<sup>a</sup> Data from one out of two experiments are reported here. Cells were sorted as in Figure 6 and cultured for 7 days in the presence of CpG and anti-CD40 mAbs. At the end of the incubation period, Ab concentrations were measured by ELISA. Titers are expressed as ELISA units (EU) as calculated by linear regression analysis plotting dilutions versus A492 OD, with titer being defined as EU of the highest dilution which give twice the absorbance of culture medium (0.1).



**FIGURE 8.** Serum Ig titers. Serum IgM (A) and total Ig (B) titers were analyzed by ELISA in blood samples harvested from 37-wk-old animals. All mice were maintained in the same animal facility. Data are expressed as mean values of five to eight mice per strain  $\pm$  SD: C57BL/6 ( $n = 8$ ), WLA ( $n = 6$ ), MBT ( $n = 6$ ), SEG ( $n = 6$ ), PWK ( $n = 5$ ), STF ( $n = 5$ ), WMP ( $n = 6$ ).

CD5 level inasmuch as anti-CD5 mAbs were able to stain naive T and activated B cells from all wild-derived strains (Figs. 1A and 2).

Altogether, these results demonstrate that all B cell subpopulations, including Bw and B-2, are able to express CD5. Therefore, the absence of CD5<sup>+</sup> B cells in wild mice cannot be explained by a defect in the CD5 pathway, even though we cannot exclude the presence of an alternative CD5 transcript that so far has only been described in human B cells (34).

Our data clearly led us to describe an original B cell population, that we have named Bw, owing to its first identification in wild-derived mice strains. This population is highly conserved between all wild-derived mice strains and all laboratory mice (Fig. 4) which is consistent with the origin of these latter. It is interesting to note that we found this Bw population both in wild-derived mice kept in animal facilities where no specific pathogen were detected (Pasteur Institute) and in conventional non-SPF conditions (Montpellier-CNRS) indicating that the composition of the gut flora does not play a specific role in the appearance of Bw cells.

The Bw cell population display an original phenotype, distinct from both B-1a, B-1b and B-2 cells. Differently to B-2, Bw cells are Mac-1 positive and express high levels of IgM and in contrast to B-1a cells, Bw cells do not express the CD5 Ag. Moreover, Bw cells express high level of both B220 and IgD as do B-2 cells and are CD9 negative and CD43 negative in contrast to both B-1a and B-1b. In this way, Bw cells cannot be classified in any of the known B cell subsets.

Bw cells are present in variable proportions depending on the strain and the organ considered. The higher frequency of Bw cells found in PWK compared with that found in STF could be explain by different numbers of progenitors or by differences in the genetic backgrounds or in their H-2 haplotype which has been described to regulate the distribution for B-1 and B-2 cells (35) or by different

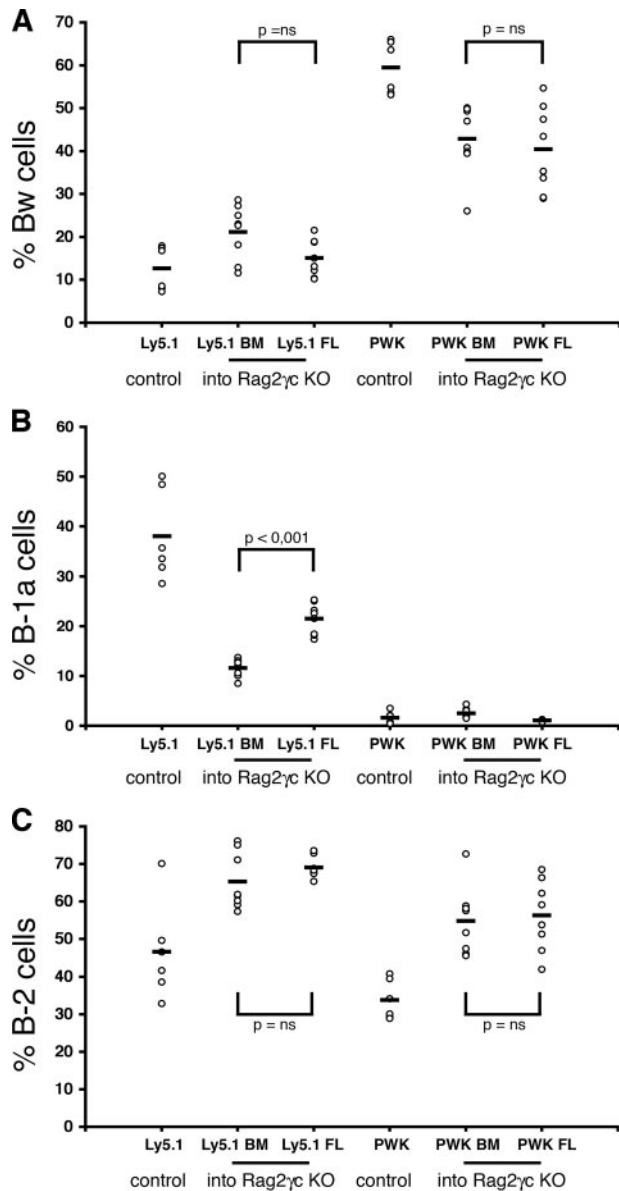
peripheral selections between these 2 strains. We have shown that for a given location, the fraction of Bw among total B cells is higher in the peritoneal cavity as compared with other organs, except for STF where they are equally distributed in the spleen and the peritoneal cavity. However, the absolute number of Bw is far greater in the spleen than in any other organs, which distinguish them from both B-1a and B-1b cells. The fact that Bw cells are present in PBL lead us to suggest that they are able to circulate between the different lymphoid organs. Rothstein et al. (36) have already highlighted phenotypic and functional differences between peritoneal and splenic B-1a subsets, leading us to speculate that peritoneal Bw cells would differ from splenic Bw cells. In this line, although they share the same phenotype, their frequency of BrMRBC specific-rosette forming cells is far much lower in spleen than in the peritoneal cavity.

Transfer experiments of either fetal liver or T- and B cell depleted bone marrow cells from PWK into Rag2  $\gamma$ c deficient mice gave rise to Bw cells but failed to differentiate in B-1a and B-1b cells. Although our data “per se” do not exclude peritoneal environmental influences, they show that Bw cell differentiation is an intrinsic property of the cell from fetal liver or adult bone marrow origin, discriminating the Bw subset from B-1 cells which do have their own progenitors enriched in the fetal liver as identify by Montecino et al. (37). Additional experiments would help us to understand whether the Bw compartment is filled in their different locations only by replenishment from adult bone marrow or also by self-replenishment as described for the B-1 subset.

In mice, natural IgM were described to be largely secreted by B-1 cells (2, 9, 10) or by MZ B cells following transfer of resting B cells in immunodeficient mice (32). In human, MZ B cells contribute to the serum IgM production (33). In both species, natural IgMs were shown to be both enriched in autoantibody specificities and to provide immediate responses against encapsulated bacteria (38, 39). Our study shows that variable levels of serum IgM and total Igs were found in the majority of the wild-derived mice, in presence or in absence of the B-1a subset. For example, SEG mice, which lack B-1 cells, secrete as much serum IgM and IgG as C57BL/6, which have B-1a cells. In contrast, WLA, which also have the B-1a population, present lower levels of both IgM and total Ig than C57BL/6. However, 2 other strains, PWK and STF, which both lack B-1 cells, do not show the canonical CD21<sup>high</sup>CD23<sup>low</sup>CD1d<sup>high</sup> phenotype of MZ B cells, are able to produce high levels of natural Igs. These data suggest that other subsets than B-1 and MZ B cells contribute to serum IgM level and therefore that Bw cells may have an important role in the production of natural Igs.

Adoptive transfers of Bw cells in lymphoid deficient mice are underway to investigate directly the role of Bw in the production of natural Igs and in protection against pathogens.

The repertoire of B-1a cells was shown to be enriched in clones specific for phosphatidylcholine, bacterial phosphorylcholine and self associated epitopes such as Thy-1 Ag (12, 40–42). High proportions of B-1a cells from laboratory mice are specific for PtC, as revealed by their ex vivo ability to form rosettes with BrMRBC (12, 13). Our data show that variable proportions of rosettes are detected with peritoneal cells from all wild-derived strains tested, even in absence of B-1 cells. Among wild-derived mouse strains lacking B-1 cells, high proportions (2% of total B cells) of BrMRBC rosettes were found in MBT (*M. musculus musculus*) and SEG (*M. spretus*), even if they were 10-fold lower than in WLA (*M. musculus domesticus*) which have the B-1a population. The frequency of BrMRBC rosettes in the peritoneal cavity of all other wild-derived strains analyzed is low but significant, suggesting that the recognition of self or neoself Ags is not restricted to



**FIGURE 9.** Fetal liver transfers. Percentage of Bw (B220<sup>high</sup>Mac-1<sup>+</sup>) (A), B-1a (CD5<sup>+</sup>IgM<sup>+</sup>) (B), and B-2 (B220<sup>high</sup>Mac-1<sup>-</sup>) (C) cells among total B cells. Irradiated alymphoid Rag2  $\gamma$ c deficient (Ly5.2) mice were reconstituted with  $7 \times 10^6$  T and B cell (CD4<sup>+</sup>CD8<sup>+</sup>CD19<sup>+</sup>)-depleted bone marrow or  $7 \times 10^6$  fetal liver cells (day = E14) from PWK or from Ly5.1 congenic C57BL/6. Six weeks after transfers, peritoneal populations analyzed by flow cytometry for CD5, IgM, Mac-1 and B220 expression. Percentage of each B cell population from Ly5.1 C57BL/6 and PWK adult mice are shown as controls. Results are from 2 experiments with a total of 6 mice per group for the controls and 8 mice per group for the transferred mice. We used the Mann-Whitney for statistical analysis. A value of  $p < 0.05$  was considered to indicate a significant difference.

B-1a cells. This conclusion is further supported by the fact that purified Bw cells from several inbred wild-derived mouse strains, cultured in the presence of CpG and anti-CD40 mAbs, were able to produce higher titers of autoantibodies than their B-2 counterparts. Moreover, the Bw/B-2 ratio of anti-tubulin, anti-actin and anti-DNA Ab titers were most often largely higher than the Bw/B-2 ratio of total Ig titers, depending of the specificity and the strain.

Bw cells can be discriminated from both B-1 and B-2 cells on the basis of Ig production and cytokine synthesis. Although B-2

cells proliferated as well as B-1 cells from C57BL/6 or as Bw cells from wild mice in response to the costimulation of CpG and anti-CD40 Abs (data not shown), Bw cells produced more anti-PC Abs, at the population level, than B-2 cells when stimulated by TLR ligands, such as LPS or CpG. It is interesting to note that depending of the wild mouse strain, anti-PC Abs express the VHT15 gene but not the IdT15 Id (data not shown). Moreover, in the presence of LPS or CpG, they do not produce more IL-6 or IL-10 than their B-2 counterparts and unlike B-1 cells they do not synthesize high levels of IL-10.

Altogether, our data show that the Bw cell population is distinct from B-1a cells by their phenotype, their presence in the whole genus *Mus* and their low ability to synthesize IL10, a “B-1 cell cytokine”. The Bw cell population is distinct from B-2 cell population by part of its phenotype, its higher ability to synthesize autoantibodies as well as Abs directed against the phosphorylcholine moiety.

The level of anti-PC Abs following stimulation by TLR ligands suggests that they may have an important role in the early response to pathogens as it is the case for MZ B cells. It has been reported that MZ B cells proliferate more efficiently than the FO B cell population in response to T independent Ags (3). At first glance our data indicate that the dichotomy observed between MZ B cells and FO B cells is not found at the level of peritoneal B-1/Bw vs B-2 proliferative responses.

In conclusion, our findings demonstrate that CD5<sup>+</sup> peritoneal B cells are not fully representative of the peritoneal B cell populations of the whole genus *Mus* and suggest that B-1a cells in laboratory mice are inherited from only one subspecies: *M. musculus domesticus*. Moreover, we identify a novel B cell population (Bw) that is highly conserved between all wild-derived and laboratory mice tested. The conservation of Bw, but not B-1 cells, during the evolution of the genus *Mus* strongly suggest that Bw cells play a key role in immunity. In the light of our findings, it would be important to investigate the presence of Bw cells in other species and particularly in human. Moreover, our data provide evidence that wild-derived mice represent a new and important experimental model to study both the development and the function of B cell subsets and their implication in innate immunity.

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## Disclosures

The authors have no financial conflict of interest.

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