Cutting Edge: Defective Follicular Exclusion of Apoptotic Antigens Due to Marginal Zone Macrophage Defects in Autoimmune BXD2 Mice

Hao Li, Qi Wu, Jun Li, PingAr Yang, Zilu Zhu, Bao Luo, Hui-Chen Hsu and John D. Mountz

*J Immunol* 2013; 190:4465-4469; Prepublished online 29 March 2013;
doi: 10.4049/jimmunol.1300041
http://www.jimmunol.org/content/190/9/4465

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/03/29/jimmunol.1300041.DC1

**References**
This article cites 23 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/190/9/4465.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Defective Follicular Exclusion of Apoptotic Antigens Due to Marginal Zone Macrophage Defects in Autoimmune BXD2 Mice

Hao Li,*† Qi Wu,* Jun Li,* PingAr Yang,* Zilu Zhu,‡ Bao Luo,* Hui-Chen Hsu,* and John D. Mountz*†‡§

Marginal zone macrophages (MZMs) act as a barrier to entry of circulating apoptotic debris into the follicles of secondary lymphoid organs. In autoimmune BXD2 mice, there is a progressive reduction in the function and numbers of MZMs. Absence of MZMs results in retention of apoptotic cell (AC) debris within the marginal zone (MZ) and increased loading of AC Ags on MZ B cells and MZ-precursor (MZ-P) B cells. The MZ-P B cells are capable of translocating the AC Ags to the follicular zone and stimulating T cells. Both MZMs and MZ-P B cells from BXD2 mice express low levels of tolerogenic signals and high levels of inflammatory signals. Thus, the current study suggests a multifaceted mechanism in which MZMs maintain tolerance to apoptotic autoantigens and suppress their translocation to follicles. Lack of clearance of apoptotic debris by MZMs drives follicular Ag–transportation by MZ-P B cells to stimulate an autoimmune response. The Journal of Immunology, 2013, 190: 4465–4469.

Apoptotic cells (ACs) contain “blebs” that are highly enriched with autoantigens, including nucleosomal DNA and small nuclear riboproteins (1, 2). Normally, the corpses of the ACs are removed locally by tissue macrophages (3). ACs and debris that escape this clearance mechanism are prevented from entering the follicles of secondary lymphoid organs by the marginal zone (MZ). There is considerable evidence that loss of integrity of the MZ barrier that results in the lack of clearance of AC autoantigens can contribute to development of autoimmune disease (4–8). The MZ macrophages (MZMs), which express the scavenger receptor SR-A and MARCO to sense and remove waste materials (8, 9), are a critical component of this barrier. In mice, autoimmune responses can be induced or promoted by either targeted deletion of SH2 domain–containing inositol 5′-phosphatase 1 (SHIP-1) in myeloid cells (9), which results in dislocations of MZMs, or treatment with an appropriate dose of clodronate liposome (CL), which results in loss of MZMs (5, 6).

BXD2 mice develop a lupus-like autoimmune disease (10). The generation of the high-affinity pathogenic autoantibodies is associated with the development of large spontaneous germinal centers (GCs) in the spleen (11–13). However, the mechanisms that permit the initial exposure of the B cells to the autoantigens have not been elucidated. The production of autoantibodies in BXD2 mice with specificity for Ags derived from a wide range of cellular compartments, including filament proteins, chromatin, and ribonuclear components (14), is consistent with the concept that autoantigens can be derived by proteolytic cleavage during the process of apoptosis (1). The present results confirm that defective clearance of apoptotic debris by MZMs and the associated transport of AC Ags by B cells can provoke a T cell response in BXD2 mice.

Materials and Methods

Mice

Wild-type C57BL/6 (B6), C57BL/6-Tg(TcraTcrb)245Chen/J (OT-II TCR transgenic [Tg]), B6-Rag2<sup>−/−</sup>, C57BL/6-Tg(CAG-OVA)916Jen/J, transgenic mice expressing the membrane-bound chicken OVA on all cell surfaces (mOVA Tg), and BXD2 recombinant inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6-Rag2<sup>−/−</sup> or B6-OT-II TCR Tg mice were backcrossed with BXD2 mice for >10 generations to generate BXD2 OT-II TCR Tg or BXD2-Rag2<sup>−/−</sup> mice, which were further intercrossed to generate BXD2 OT-II TCR Tg Rag2<sup>−/−</sup> mice. Apoptotic cells

For in vivo administration of ACs, mice were injected i.v. with 2 × 10<sup>7</sup> apoptotic thymocytes. For depletion of MZMs, mice were treated i.v. with 150 μg CL (Encapsula NanoSciences, Nashville, TN) 4 h before AC transfer, as described (5). Control mice were treated with PBS liposomes. The dose for selective delivery to MZMs was optimized using Fluoroliposome (Encapsula NanoSciences).

Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294; †Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294; ‡Division of Hematology and Oncology, Department of Medicine, University of Alabama, Birmingham, AL 35294; and §Birmingham VA Medical Center, Birmingham, AL 35294

Received for publication January 17, 2013. Accepted for publication March 1, 2013.

This work was supported by a VA Merit Review Grant (101BX00600-01), as well as grants from the National Institutes of Health/National Institute of Allergy and Infectious Disease (1AI 071110, ARRA 3RO1AI71110-02S1, and 1RO1 AI083705), the American College of Rheumatology and Education Foundation, the Arthritis Foundation, and the Lupus Research Institute. Flow cytometry and confocal imaging were carried out at the University of Alabama at Birmingham Comprehensive Flow Cytometry Core (P30 AR048311 and P30 AI027767) and University of Alabama at Birmingham Analytic Imaging and Immunoragents Core (P30 AR048311), respectively.

Address correspondence and reprint requests to Dr. Hui-Chen Hsu and Dr. John D. Mountz, Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Room 307, Shelby Building, 1825 University Boulevard, Birmingham, AL 35294-0007. E-mail addresses: rheu078@uab.edu (H.-C.H.) and jdmountz@uab.edu (J.D.M.)

The online version of this article contains supplemental material.

Abbreviations used in this article: AC, apoptotic cell; CL, clodronate liposome; FO, follicular; GC, germinal center; mOVA, membrane-bound chicken OVA; MZ, marginal zone; MZM, marginal zone macrophage; MZ-P, marginal zone precursor; RPM, red pulp macrophage; SHIP-1, SH2 domain–containing inositol 5′-phosphatase 1; Tg, transgenic.
CD4 T cell stimulation

Splenic CD4 cells from BXD2 OT-II TCR Tg, Rag2−/− mice were labeled with CFSE (5 μM) and transplanted i.v. into recipient mice (2 × 10^6/mouse). Recipient mice were administered apoptotic thymocytes prepared from mOVA Tg mice 12 h later and sacrificed after an additional 72 h. In some experiments, red pul prep macrophages (RPMs) (F4/80^+CD11b^+) or follicular (FO; CD19^+IgM^+CD21^hiCD23^lo), MZ (CD19^+IgM^+CD21^hiCD23^hi), or MZ-precursor (MZ-P; CD19^+IgM^+CD21^hiCD23^lo) B cells were FACS sorted from mice that were injected with mOVA’ Ags 30 min prior to sacrifice. In other experiments, sorted RPMs or FO, MZ, or MZ-P B cells (5 × 10^6 each) from naive 2-mo-old BXD2 mice were cocultured with purified and CFSE (1 μM)-labeled OT-II CD4 T cells (2 × 10^5) in the presence or absence of either OVA protein or OVA323-339 peptide, as described previously (15). In all in vitro experiments, CD4 T cell proliferative response was determined by the reduction in CFSE intensity by flow cytometry 3 d after coculture.

Reagents and analyses

Flow cytometry and immunofluorescence microscopy were carried out as previously described (12). Real-time quantitative PCR was carried out, as described (12), using the following primers: Il6, 5'-TCCAATTTTGGTAGCATCATCAGTC-3' (forward), 5'-CAGACTGCACTCAACG-3' (reverse); Il10, 5'-AATTCCTCTGTCAGAGGACG-3' (forward), 5'-TGCTAGTTTGATGATGAAATCAG-3' (reverse); and Tgf, 5'-CTACTATGCTAAAGAGTGAAGG-3' (forward), 5'-CATGTTGCTCCACCTTGGTTG-3' (reverse). For Western blot analysis, anti-Btk (Santa Cruz), anti-IDO (Abcam), anti-GAPDH, anti-SHIP1, and anti-rabbit HRP-conjugated Ab (Cell Signaling Technology) were used.

Results and Discussion

Reduction of MZMs in BXD2 mice

Confocal imaging (Fig. 1A) and FACS analysis (Fig. 1B) were used to determine the integrity and enumerate the percentage of SIGN-R1^+ MZMs in the spleens of autoimmune BXD2 mice. There was an age-dependent loss of MZMs in the BXD2 mice but not in the B6 mice (Fig. 1A, 1B). The total numbers of F4/80^+CD11b^+I-A^b^SIGN-R1^+ MZMs (16) were equivalent in 3-mo-old B6 and BXD2 mice, but there was a dramatic loss of these cells in 9-mo-old BXD2 mice but not B6 mice (Fig. 1B). There was an age-related defect in the clearance of ACs, as indicated by an increase in the number of TUNEL^+ ACs found near the MZMs and inside areas of the follicle distinct from the GCs in BXD2 mice (Fig. 1C). Consistent with these, there were age-related increases in the levels of circulating autoantibodies for MARCO, SR-A, and DNA in BXD2 mice (Fig. 1D). Elevation of IgG anti-MARCO and anti-SR-A was found in the serum of lupus patients and mouse models of lupus; these autoantibodies potentially affect macrophage phagocytosis of ACs (7).

Capture of AC debris by B cells and their intrafollicular migration

The functional activities of the MZMs were evaluated by the ability to clear exogenous CFSE-labeled apoptotic thymocytes (Fig. 2A). Compared with B6 mice, the apoptotic thymocytes persisted for an extended period of time in 2-mo-old BXD2 mice. Thus, the functional defects in the MZMs precede the loss of these cells and represent one of the earliest autoimmune defects detected in the BXD2 strain.

However, the failure to clear Ags alone is not sufficient to induce an autoimmune response in BXD2 mice, because large GCs are found inside the follicles. Cyster and colleagues (15) showed that B cells can shuttle between the MZ and FO areas, thereby facilitating Ag transport. We established previously that, in BXD2 mice, there is the expansion in number of MZ-P B cells that is associated with the reduction in the MZ B cells and the heightened Ag delivery by MZ-P B cells (17, 18).

To determine whether BXD2 B cells can capture and deliver Ags in MZM-deficient conditions, we tracked CFSE-labeled apoptotic thymocytes in mice that were treated with 150 μg of CL. This dose was used because it selectively induced uptake of liposomes by MZMs (Supplemental Fig. 1A). In mice administered CL, there was a time-dependent capture of Ags by MZ B and MZ-P B cells but not FO B cells (Fig. 2B, Supplemental Fig. 1B). The numbers of CFSE^+ cells, which were located predominantly in the MZ, peaked at 5–15 min, and the majority was processed at 60 min (Fig. 2C, left panel). This may be associated with the higher uptake of CFSE by MZ B cells compared with MZ-P B cells during these early time points (Fig. 2B, Supplemental Fig. 1B). Within 5–30 min, there was a rapid migration of MZ-P B cells to the periphery of the follicle and into the MZ (Fig. 2C, right panel, Supplemental Fig. 1C, 1D). By 1 h after
administration of the ACs, the majority of MZ-P B cells, but not MZ B cells, exhibited massive inward migration into the follicles. At this time point, the majority of both the MZ-P and MZ B cells carried the apoptotic debris (Fig. 2B, 2C, Supplemental Fig. 1B).

Apoptotic Ags capturing MZ-P B cells stimulate CD4+ T cells

To determine whether MZMs are mechanistically required to suppress the T cell response to ACs and associated Ags, we transferred CFSE-labeled OT-II TCR Tg T cells into BXD2 and B6 mice and assessed the response of Vβ5+ T cells to apoptotic thymocytes derived from mOVA Tg mice. Significantly higher numbers of OT-II T cells entered the cell cycle in the spleens of the 2- and 9-mo-old BXD2 mice than the spleens of age-matched B6 mice postapoptotic mOVA+ thymocyte transfer (Fig. 3A, right panel). The percentages of CFSE-labeled OT-II T cells that entered the cell cycle in mice administered CL were similar to that observed in 9-mo-old BXD2 mice (Fig. 3A, right panel), which is consistent with the dramatic loss of MZMs observed in the older BXD2 mice (Fig. 1A, 1B).

We showed previously that the T cell costimulatory function of MZ-P B cells is superior to other B cells in BXD2 mice (17, 18). Therefore, we cocultured CFSE-labeled OT-II CD4+ T cells with F4/80+ RPMs or subpopulations of B cells sorted in the spleens of 2-mo-old BXD2 mice. (A) Confocal imaging showing the kinetics of clearance of administered CFSE-labeled apoptotic thymocytes (pseudocolor red) in the MZ area in the spleen of 2-mo-old mice (left panel). High-power view of the indicated (box) areas (right panel). Original magnification ×200. (B and C) BXD2 mice (2 mo) were treated with either CL- or PBS-liposome 4 h prior to CFSE-labeled apoptotic thymocyte administration. Following AC administration, mice were sacrificed at the indicated time points. (B) FACS analysis of subpopulations of B cells that were positive for AC Ags (CFSE+) (left panel), FO, MZ, and MZ-P B cells were gated as described (17, 18). Confocal imaging assessment of the binding of FACS-sorted MZ or MZ-P B cells with CFSE+ (pseudocolor red) apoptotic debris (right panel). A representative cell from the indicated subset is shown. Original magnification ×1000. (C) Confocal imaging showing a representative spleen follicle from each group to reveal the clearance of the administered CFSE+ apoptotic thymocytes (pseudocolor red) in the spleen (left panel) and the anatomic location of FO (red: CD1d+CD23+), MZ (green: CD1d+CD23-), MZ-P (yellow: CD1d+CD23+), and GC (blue: PNA+) in each group (right panel). Original magnification ×200. (n = 2–3 mice/group for three independent experiments).

FIGURE 3. Stimulation of CD4 T cells by MZ-P B cells carrying AC Ags. (A) Flow cytometry analysis of the percentage of the transferred BXD2 OT-II CD4+ T cells (TCR Vβ5) in total spleen cells in the indicated recipient groups (left panels). Flow cytometry analysis of the in vivo OVA Ag-specific proliferative response of the transferred OT-II TCR CD4+ T cells, indicated by attenuation of CFSE intensity (right panels). Control mice (2 mo old) were administered CL. (B) Bar graph represents the in vitro proliferative response of OT-II TCR CD4+ T cells (CFSElo) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA+ thymocytes. (C) Flow cytometry analysis of the in vitro OT-II TCR CD4+ T cells, indicated by attenuation of CFSE intensity (right panel). Control mice (2 mo old) were administered CL. (B) Bar graph represents the in vitro proliferative response of OT-II TCR CD4+ T cells (CFSElo) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA+ thymocytes. (C) Flow cytometry analysis of the in vitro OT-II TCR CD4+ T cells, indicated by attenuation of CFSE intensity (right panel). Control mice (2 mo old) were administered CL. (B) Bar graph represents the in vitro proliferative response of OT-II TCR CD4+ T cells (CFSElo) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA+ thymocytes. (C) Flow cytometry analysis of the in vitro OT-II TCR CD4+ T cells, indicated by attenuation of CFSE intensity (right panel). Control mice (2 mo old) were administered CL. (B) Bar graph represents the in vitro proliferative response of OT-II TCR CD4+ T cells (CFSElo) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA+ thymocytes. (C) Flow cytometry analysis of the in vitro OT-II TCR CD4+ T cells, indicated by attenuation of CFSE intensity (right panel). Control mice (2 mo old) were administered CL. (B) Bar graph represents the in vitro proliferative response of OT-II TCR CD4+ T cells (CFSElo) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA+ thymocytes.
from the spleens of BXD2 mice that had been administered mOVA thymocytes. These results show that both MZ and MZ-P B cells can present Ags derived from ACs, and this Ag presentation and stimulation of T cells are higher in BXD2 mice compared with B6 mice (Fig. 3B, Supplemental Fig. 2). Although the ability of MZ-P B cells to process intact OVA proteins for stimulation of CD4+ T cells was lower than that of RPMs, the ability of MZ-P B cells to present OVA peptide for stimulation of T cells was superior to that of all other tested B cell populations from BXD2 spleens (Fig. 3C). This suggests that MZ-P B cells that have migrated into the FO region may either directly process or capture processed AC Ags, thereby stimulating CD4+ T cells.

Under normal conditions, phagocytic cells that ingest ACs produce tolerogenic signals, including TGF-β and IL-10, which maintain tolerance to self-Ags (19). Both MZMs and MZ-P B cells from 3-mo-old BXD2 mice exhibited lower expression of genes encoding tolerogenic cytokines, including TGF-β and IL-10, and higher expression of the gene encoding the immunogenic cytokine IL-6 compared with those from B6 MZMs and MZ-P B cells (Fig. 3D). IDO was recently shown to be an important tolerogenic signal inducer in MZMs of B6 mice following AC administration (6). However, IDO was detectable in MZMs of unmanipulated younger MRL-Fas+/+ mice, and its inhibition with D-1-methyl-tryptophan promoted autoimmunity (6), suggesting that low IDO can enhance, but is not the primary cause of, autoimmunity in MRL-Fas+/+ mice. In contrast, to our knowledge, the current study demonstrated for the first time that CD11b+ cells from a mouse model of spontaneous autoimmunity expressed significantly lower levels of IDO compared with those from normal B6 mice (Fig. 3E). Also, local high levels of IL-6 in the MZ area might contribute to an immunogenic signal for AC Ags, because IL-6 degrades IDO (20). The TGF-β–IDO+ITIMs stimulate SHIP-1 (Innp54d), and the TGF-β–IDO–SHIP axis was recently identified as the major long-term tolerogenic signal in DCs (21). There also were dramatically lower levels of SHIP-1, together with higher levels of its counteracting molecule, Btk (9), in the CD11b+ cells from the spleens of BXD2 mice (Fig. 3E). These findings are consistent with the observations that loss of the expression of SHIP-1 in myeloid cells of Lyz.Cre Ship1−/− mice was associated with disrupted MZM integrity and enhanced splenomegaly and that deficiency of Btk in Lyz.Cre Ship1−/− mice reversed these phenotypes (9).

In summary, we showed previously that the development of the high titers of autoantibodies in BXD2 mice is dependent on abnormalities in two compartments in the spleen: enhanced production of type I IFNs in the MZ induces FO translocation of Ag-bearing MZ-P B cells, which costimulate T cells (17, 18), and enhanced IL-17 production in the GCs that upregulates regulator of G-protein signaling proteins, thereby enhancing T–B cell interactions (12, 22). We now have observed that these abnormalities are preceded by the development of defects in the MZ barrier in BXD2 mice. Collectively, the present results suggest a novel mechanism of autoimmune in which “leaks” in the FO exclusion of self-apoptotic Ags, together with enhanced activity of carrier B cells, subverts otherwise protected adaptive immune processes in the follicles. McHeyzer-Williams et al. (23) elegantly described the importance of B cells as initial Ag-transporting cells in adaptive immunity. The present study directly demonstrated that, in a situation in which MZM apoptotic debris ingestion and tolerogenic functions are defective, FO translocating immunogenic B cells can capture and deliver the autoantigens into the follicles, thus providing the previously unknown bridging mechanism between defective clearance of apoptotic debris and the development of autoantibodies. Strategies that restore and retolerize MZM function may represent a novel approach to attenuation of autoimmune disease.

Acknowledgments

We thank Dr. Fiona Hunter for review of the manuscript and Karen Beeching for excellent secretarial assistance.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Information

Supplemental Figure 1. Clodronate liposome depletion of MZMs enhanced apoptotic debris captured by B cells. (A) A dose dependent response of liposomes uptake by MZMs. BXD2 mice (2-mo) were injected with 10, 30 or 200 μl (equivalent to 50, 150, or 1000 mg CL, respectively) of Dil (a lipophilic fluorescent dye: em ~580 nm) containing liposomes. Mice were sacrificed at the indicated time points. Frozen sections of the spleens were stained with an anti-MARCO antibody. Confocal imaging analysis was used to determine the distribution and uptake of Dil (red) by MARCO+ MZMs (green). Double positive cells are yellow color. (B, C) BXD2 mice (2-mo) were treated with CL (150 μg per mouse) 4 hrs prior to CFSE-labeled apoptotic thymocyte (AC+: 2x10^7 cells/mouse) administration. Following AC administration, mice were sacrificed at the indicated time points (min). CFSE+ apoptotic debris captured by B cells in the absence of MZMs. BXD2 mice (2-mo-old), treated with CL to deplete MZMs, were administered CFSE labeled apoptotic thymocytes (AC) at the indicated times (min) prior to sacrifice. Control mice were either treated with PBS liposomes or were not administered apoptotic thymocytes (AC–). (B) Single cell suspension from the spleen was labeled with fluorochrome-labeled antibodies against CD19, IgM, CD21, and CD23. Cells were subjected to flow cytometry to enumerate the percentage of FO (IgM^lo^CD21^lo^CD23^hi^), MZ (IgM^hi^CD21^hi^CD23^lo^), or MZ-P (IgM^hi^CD21^hi^CD23^hi^) CD19+ B cells that were positive for apoptotic cells (CFSE–) (NA, not applicable; ND, not detectable; *p<0.05, **p<0.01, ***p<0.005 between the indicated comparisons; N=2-3 mice per group for 3 independent experiments). (C) Enlarged view of a representative spleen follicle from the indicated group to reveal the anatomic location of FO (red: CD1d^−^CD23^+^), MZ (green: CD1d^+^CD23^−^), MZ-P (yellow: CD1d^+^CD23^hi^), and GC (blue: PNA^+^) at the indicated times. All groups of mice were administered CL. Arrows indicate the area where the majority of MZ-P B cells are localized. (D) Quantitative analysis of the percent of CD23^hi^CD1d^+^ (yellow) B cells in the inner 1/2 of a spleen follicle (**p<0.01, ***p<0.005 between the indicated comparisons; N=2-3 mice per group for 3 independent experiments).

Supplemental Figure 2. Stimulation of CD4 T cells by B cells carrying apoptotic cell antigens. Flow cytometry analysis of the in vitro OT-II T-cell proliferative response (CFSE^lo^) to OVA antigens presented by the indicated cell populations. The indicated mOVA antigen bearing APCs were FACS sorted from the spleens of mice that have been administered 2x10^7 apoptotic mOVA+ thymocytes 30 min prior to sacrifice. A group of 2-mo-old mice were treated i.v. with 150 μg of CL 4 h before administration of apoptotic cells. Sorted mOVA bearing APCs (5 x 10^5) were cocultured with purified and CFSE (1 μM) labeled OT-II CD4 T cells (2 x 10^5). CD4 T cell proliferative response was determined by the reduction of CFSE intensity by flow cytometry 3 d after coculture. RPMs, FO, MZ, and MZP B cells were sorted as F4/80^lo^, CD19^IgM^lo^CD21^lo^CD23^hi^, CD19^IgM^hi^CD21^hi^CD23^lo^, or CD19^IgM^hi^CD21^hi^CD23^hi^ cells, respectively (N=4 per group; *p < 0.05; **p < 0.01; ***p < 0.005 compared with the same population of cells from B6 mice).