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Hematopoietic stem and multipotent progenitor cells produce IL-17, IL-21 and other cytokines in response to TLR signals associated with late apoptotic products and augment memory Th17 and Tc17 cells in the bone marrow of normal and lupus mice

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Abstract

We studied effects of early and late apoptotic (necroptotic) cell products, related damage associated alarmins and TLR agonists, on hematopoietic stem and progenitor cells (HSPC).¹ Surprisingly, normal HSPC themselves produced IL-17 and IL-21 after 1½ days of stimulation, and the best stimulators were TLR7/8 agonist; HMGB1-DNA; TLR9 agonist, and necroptotic B cells. The stimulated HSPC expressed additional cytokines/mediators, directly causing rapid expansion of IL-17⁺ memory CD4 T (Th17), and CD8 T (Tc17) cells, and antigen-experienced IL-17⁺ T cells with “naïve” phenotype. In lupus marrow, HSPC were spontaneously pre-stimulated by endogenous signals to produce IL-17 and IL-21. In contrast to HSPC, megakaryocyte progenitors (MKP) did not produce IL-17, and unlike HSPC, they could process and present particulate apoptotic autoantigens to augment autoimmune memory Th17 response. Thus abnormally stimulated primitive hematopoietic progenitors augment expansion of IL-17 producing immune and autoimmune memory T cells in the bone marrow, which may affect central tolerance.

¹Abbreviations

HSPC, hematopoietic stem and progenitor cells; LSK, Lineage[−]Sca-1⁺cKit⁺ cells; LSKen, LSK-enriched cells; LT-HSC, long-term hematopoietic stem cells; ST-HSC, short-term hematopoietic stem cells; MPP, multipotent progenitor cells; MKP, megakaryocyte progenitor cells; MEP, bi-potent megakaryocyte- and megakaryocyte-erythroid progenitor cells; MM, MKP and MEP cells; MDP, macrophage dendritic cell progenitors; DAMP, damage associated molecular pattern; B/WFI, (NZB × NZW)F1; TCM, central memory T cells; TEM, effector memory T cells; TSCM, stem cell memory T cells; HMGB1, High-mobility group box-1; ssDNA, single-strand DNA.

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Keywords

Hematopoietic progenitors; Memory Th17/Tc17 cells; Apoptosis; TLR signals; Cytokines; Lupus; Bone marrow

1. INTRODUCTION

Autoimmune inflammatory diseases, especially lupus, is associated with impaired disposal of apoptotic cell products due to deficiencies of scavenging molecules in phagocytic cells, such as Marco and other scavenger receptors, or complement components such as C1q which facilitate phagocytosis of apoptotic cells [1-6]. Accumulated apoptotic products, such as HMGB1, nucleosomes, DNA or RNA act as *endogenous* TLR ligands, abnormally stimulating cells of the innate and adaptive immune system [1, 2, 5-21]. For instance, the non-histone chromosomal protein HMGB1 released from defectively cleared apoptotic cells forms highly inflammatory complexes with DNA or nucleosomes to stimulate immune cells via TLR 4, RAGE and TLR 2 on the cell surface, or TLR9 in the endosome/lysosome via DNA [7, 11, 21]. Similarly, nucleosomes containing DNA, or ribonucleoproteins containing RNA can stimulate cells of the innate immune system by TLR9 or by TLR 7/8 and TLR 3 respectively [16-20].

In the bone marrow, selection of developing B cells is associated with extensive apoptosis [22], but it is unknown what effect the apoptotic products would have there if not cleared properly. In situations associated with extramedullary hematopoiesis, such as lupus, we showed previously that megakaryocyte progenitors (MKP), mobilized or generated in the periphery, can process and present apoptotic autoantigens like professional APC to induce and augment Th17 and the doubly potent Th1/Th17 responses [10, 23]. However, the effect of such apoptotic products on the earliest hematopoietic stem and progenitor cells (HSPC) is unknown. HSPC express TLRs [24-29], but so far, studies have focused on exogenous TLR 4 and TLR 2 ligands derived from pathogens, and investigated extrinsic effects of cytokines systemically produced by the TLR-stimulated immune system of the infected host, which secondarily affected the HSPC.

Herein, we examined the effect of endogenous apoptotic cell products and related TLR ligands on HSPC from normal and lupus prone mice. The HSPC are Lineage⁻Sca-1⁺cKit⁺ (LSK) cells consisting of long-term and short-term hematopoietic stem cells (LT-HSC and ST-HSC), and multipotent progenitors (MPP). However, interpreting the responses of lupus HSPC to the apoptotic TLR agonists, in contrast to their normal counterparts, is problematic because of the confounding effects of inflammatory cytokines and chemokines produced systemically that modify the behavior of HSPC in a systemic autoimmune inflammatory disease like lupus. The status of HSPC in the bone marrow of the lupus mice is not static, as they are constantly being stimulated (and exhausted) by exogenous cytokines, such as IL-1, IL-6, GM-CSF, IFN α , as well as being exposed to defectively cleared apoptotic products and they are also being mobilized out of the bone marrow to sites of extramedullary hematopoiesis [10, 23]. Therefore, we relied on the bone marrow HSPC from normal mice to determine how they would respond to apoptotic cells/products, such as apoptotic B cells,

apoptotic thymocytes, necrotic (necroptotic) B cells, HMGB1-DNA complex, or nucleosomes; as well as, surrogate TLR agonists that are involved in stimulation by late apoptotic products' inflammatory signals, namely, Poly (I:C), LPS, R848 or CpG1585, which stimulate TLR 3, 4, 7/8 and 9 respectively. We found that after 1½ days of culture, endogenous apoptotic products and related TLR ligands unexpectedly caused production of IL-17 and IL-21 by HSPC themselves, although the cytokine producing HSPC at that time after culture had still retained their primitive stem and progenitor cell surface markers. Furthermore, we found that the stimulated HSPC expressed mRNA for additional cytokines and signals that were associated with rapid expansion of IL-17 producing CD4 T (Th17), and CD8 T (Tc17) memory T cells in the marrow within 1½ days of culture in vitro, without requiring polarizing conditions. In contrast to the normal mice, HSPC from lupus prone mice were already pre-stimulated by endogenous factors as mentioned above, and any further stimulation by the apoptotic TLR agonists ex vivo yielded a muted response. In contrast to HSPC, MKP in the marrow did not produce IL-17 when presented with apoptotic cell products, but they induced an expansion of autoimmune Th17 cells in lupus mice by processing and presenting apoptotic nucleosome particles. HSPC, unlike MKP do not have phagocytic ability or APC function [23, 29]. Thus apoptotic product stimulated HSPC and MKP augment IL-17⁺ autoimmune memory T cells in the bone marrow by different mechanisms.

2. MATERIALS AND METHODS

2.1. Mice

NZB × NZWF1/J (B/WF1), C57BL/6J and C57BL/6-II17a^{tm1Bcgen}/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six to 10 weeks old females were used, and all studies were approved by the institutional animal care and use committee.

2.2. Cell isolation

Tibias and femurs were separated from mice (2 tibias and 2 femurs per mice) after removing skin, muscle, lymph nodes, ligaments, and peripheral blood contamination. Bone marrow cells pooled from 10 animals/experiment were collected by flushing bones with complete RPMI through a syringe with 25-gauge needle, and then single cell suspensions were prepared after RBC lysis. Splenocyte single cell suspension was prepared by digestion with 1mg/mL Collagenase IV (Worthington Biochemical, Lakewood, NJ) and 50µg/mL DNase I (Roche Applied Science, Indianapolis, IN), followed by RBC lysis. Lin⁻Sca-1⁺cKit⁺ enriched cells (LSKen) were purified with some variations depending on the aim of the experiments, as detailed in “Results” Section 3.1.1, and as explained there, commercially available LSK isolation kits, or Flow Cytometry-sorting were not suitable for our functional studies with large numbers of LSK cells that also required supporting stromal cells from the natural bone marrow environment. Usually, the following cell types were depleted (and saved) stepwise from bone marrow using magnetic bead-conjugated B220, CD11b, and then rabbit anti-mouse CX3CR1 IgG (ProSci, Poway, CA) followed by anti-rabbit IgG magnetic beads (Miltenyi Biotec, Auburn, CA). After depletion of B220⁺, CD11b⁺, and CX3CR1⁺ cells, LSKen cells were then isolated by positive selection using anti-CD117 and anti-Sca-1 magnetic beads or just anti-Sca-1 beads and then gating in flow cytometer for CD117⁺ and

Sca-1⁺ cells, depending on the experiment (Section 3). Further purified LSK cells were isolated by depletions of B220⁺, CD11b⁺, CD3⁺, CX3CR1⁺ and CD41⁺ cells, followed by positive selection by anti-CD117 and anti-Sca-1 magnetic beads. MKP/MEP cells (megakaryocyte progenitor/ bi-potent megakaryocyte-erythroid progenitors, called MM cells here) were isolated by anti-CD41 and anti-CD117 beads in the last positive selection step from aliquots of LSK-enriched cell preparation at its penultimate purification step. Splenic T cells were isolated using magnetic beads conjugated CD90.2 or pan T cell isolation kit (Miltenyi Biotec). For obtaining flow cytometry-sorted bone marrow or splenic CD41⁺CD117⁺ cells; CD117⁺ cells were first isolated using anti-CD117 magnetic beads (Miltenyi Biotec). The CD117⁺ cells were then stained with FITC-anti-CD41 and sorted for CD41⁺ cells by LSRII (BD, San Jose, CA) at Northwestern University Robert H. Lurie Comprehensive Cancer Center core facility.

2.3. Flow cytometry

Flow cytometry antibodies were purchased from eBioscience (San Diego, CA) unless otherwise specified. LSK cells that had been cultured with or without TLR agonists were analyzed by staining cells with Fixable Viability Dye eFluor[®] 506, BV421-anti-CD117 (BD Pharmingen, San Jose, CA), FITC-anti-Sca-1, PE-anti-Flk2, PerCPy5.5-anti-CD11b, PE-Cy7-anti-CD3/B220, APC-eFluor[®] 780-anti-CD90.2 and Alexa Fluor 700[®]-anti-CD34. Phenotyping of multiple T cell subsets was done by staining with Fixable Viability Dye eFluor[®] 506, CD117-BV421 (BD Pharmingen), Alexa Fluor 700[®]-anti-CD8a and Mouse Naïve/Memory T cell panel kit (BD Pharmingen), including PE-anti-CD44, PerCP-Cy[™]5.5-anti-CD4, allophycocyanin-anti-CD62L and APC-Cy[™]7-anti-CD3. For intracellular staining of IL-17 and IL-21, cultured cells were incubated with 50ng/ml PMA, 500ng/ml ionomycin and 1× GolgiPlug-Brefeldin A solution at 37°C for 4 hrs. Cultured cells were then harvested, surface stained with cell markers, fixed and permeabilized in fixation/permeabilization buffer (eBioscience) and stained for allophycocyanin- or FITC-conjugated anti-IL-17 or IL-21. Cells were acquired by LSRII with FACSDiva (BD, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR). Flow cytometry positively stained cell gating was based on fluorescence-minus-one (FMO) samples and biological control samples.

2.4. Apoptosis related TLR agonist stimulation

TLR agonists that are related to inflammatory signal of late apoptotic products, directed at TLR 3, TLR 4, TLR 7/8, and TLR 9 were used for stimulating LSK cells. LSK cells from both normal or lupus prone mice were fractionated by magnetic beads and cultured in triplicate with TLR 3, 4, 7/8 or 9 ligands (InvivoGen, San Diego, CA), which were respectively, Poly (I:C) 10µg/mL, LPS 200ng/mL, R848, 1µM, and CpG oligonucleotides 1585, 5µg/mL, for 36 hours [30, 31]. After TLR ligand stimulation, cultured cells were harvested and stained for LSK cell surface markers, followed by intracellular staining of IL-17 and IL-21 and examined by flow cytometry. The culture time for 36 hours was optimal for the cytokine detection by ICS, as 24 hr. culture was too early, and many cells died after stimulation in 48 hr cultures.

2.5. Apoptotic cell product stimulation

For *in vitro* apoptotic cell product stimulation, LSKen cells were cultured with either apoptotic thymocytes (ratio of LSKen: apoptotic thymocytes = 1:20) [10], apoptotic B cells (1:20), necrotic (necroptotic) B cells (ratio of LSKen: necrotic B cells = 1:20) [10], nucleosomes 5µg/mL, HMGB1 100ng/mL [32], or HMGB1-DNA complex (20ng/mL ssDNA with 100ng/mL HMGB1) [33, 34]. Cells were stimulated with apoptotic cell products for 36 hours, and IL-17 and IL-21 production was examined by flow cytometry. Apoptotic thymocytes were produced by incubating thymocytes with 0.5µg/mL staurosporine (Sigma-Aldrich, St. Louis, MO) at 37°C incubator for 6 hrs [35], followed by 2 washes of cold cRPMI. Apoptotic B cells were prepared by osmotic shock, and necrotic (necroptotic) B cells were prepared by five freezing-thawing cycles of isolated bone marrow B220⁺ cells [36]. Nucleosomes were isolated from chicken erythrocyte nuclei [9]. ssDNA bound HMGB1 was synthesized by incubating denatured sonicated calf thymus DNA (Sigma-Aldrich) with recombinant mouse high-mobility group box-1 (HMGB1) protein (eBioscience) [33, 34]. Briefly, calf thymus DNA was resuspended in nuclease-free water and 200bp DNA fragments were obtained by sonication. For binding ssDNA with HMGB1, 200ng of sonicated calf thymus DNA fragments were suspended in 50µL TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and denatured at 100°C for 3 min. The ssDNA was then quickly mixed with 20µL of 10× buffer (500mM NaCl, 150mM Tris-HCl pH 7.5, 10mM DTT, 1mM EDTA, 1% Triton X-100) and 1µg HMGB1, to a final volume of 150µL. The HMGB1-DNA complex was formed by incubating the mixture at 37°C for 30 min and stored at 4°C until use.

2.6. Cell cycle Status of stimulated LSK cells

LSK cell cycling status was determined by Ki-67 and DAPI staining. LSKen cells were isolated by magnetic beads and stimulated with R848 at 37°C for 36 hrs. After stimulation, cultured cells were surface stained with LSK cell markers, then fixed and permeabilized by using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained with PE-eFluor® 610-anti-Ki-67 (eBioscience). After intracellular staining of Ki-67, cells were incubated with 10µg/mL DAPI (Sigma-Aldrich) for 20 mins at room temperature before being acquired by flow cytometer to determine cell cycle status [37].

2.7. qRT-PCR for measurement of mRNA for cytokines and mediators

Purified LSK cells from normal and lupus prone mice were cultured with R848 or CpG1585 for 16 hours. Unstimulated B220⁺ cells from normal mice served as control. Total RNA from the cultured cells was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) with DNase treatment, and 1.25µg of total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed in triplicates using TaqMan Gene Expression Master Mix and a 7300 Fast Real-time PCR System (Applied Biosystems, Grand Island, NY). Expression of the tested genes was normalized relative to the levels of GAPDH. The relative expression levels were calculated using the 2^{-C_t} threshold cycle method, as described [23].

2.8. Confirmation of IL-17 production by LSK using GFP reporter mice

To confirm intracellular IL-17 staining results in stimulated LSK cells in response to apoptotic cell signals, LSKen cells from C57BL/6J mice and C57BL/6-Il17a^{tm1Bcgen}/J GFP reporter mice were cultured in triplicate with either necrotic B cells or R848. After 36 hrs of stimulation, cells were stained for LSK cell surface markers and examined by flow cytometry. IL-17-GFP signal of antigen-stimulated LSKen cells was determined by comparing with fluorescence intensity of unstimulated LSKen cells from GFP reporter mice and stimulated LSKen cells from normal mice.

2.9. Confirmation of IL-17 response in LSK cells by ROR γ (t) expression

Bone marrow LSK cell's ROR γ (t) expression was examined to confirm their IL-17 producing ability. LSKen cells from C57BL/6J mice were cultured with either necrotic B cells or R848 for 36 hrs. Cultured cells were stained for LSK cell surface markers, followed by fixation and permeabilization using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience), then intracellularly stained with allophycocyanin-anti-ROR γ (t) (eBioscience).

2.10. Analysis of memory Th17 and IL-17 producing CD8 (Tc17) cells in bone marrow

To study the effect of stimulated HSPC in expanding memory Th17 and CD8 Tc17 cells, bone marrow LSKen (B220⁻CD11b⁻CD90⁻CX3CR1⁻CD41⁻CD117⁺) or LSK cells (B220⁻CD11b⁻CD90⁻CX3CR1⁻CD41⁻CD117⁺Sca-1⁺) from normal mice were cultured in triplicate with bone marrow CD90.2⁺ T cells in the presence of necrotic B cells, nucleosomes, CpG1585 or R848 for 36 hrs. To further study HSPC's effect on memory Th17 and CD8 Tc17 cell expansion with apoptotic cell antigens processed and presented by antigen presenting cells, bone marrow MM cells mixed with LSKen cells from lupus mice were cultured in triplicate with bone marrow CD90.2⁺ T cells in the presence of necrotic B cells or nucleosomes for 36 hrs. After *in vitro* stimulation, cells were then harvested, stained for Naïve/Memory T cell subset markers and intracellular IL-17, and acquired for flow cytometry.

2.11. ELISPOT assay

ELISPOT assay plates (Millipore, Billerica, Massachusetts) were coated with anti-IL-17 capture Ab (BD Pharmingen) in PBS at 4°C overnight. Splenic T cells (1×10^6) were cultured with flow cytometry sorted bone marrow and splenic CD41⁺CD117⁺ MM cells, CD41⁻CD117⁺ cells, bone marrow CD11b⁺ cells and splenic CD11c⁺ cells in the presence of 10 μ g/mL nucleosomes. Cells were removed after 48 h, and the reactions were visualized by addition of anti-IL-17 Ab biotin, followed by enzyme conjugate streptavidin-HRP and subsequent AEC substrate (3-amino-9-ethyl-carbazole; Sigma-Aldrich). IL-17-expressing cells were detected by Immunospot scanning and analysis (Cellular Technology Ltd, Shaker Heights, OH). IFN- γ ELISPOT were also done, as described [10].

2.12. Statistical analysis

The Student two-tailed t tests were used for comparative analysis of results from each apoptotic TLR agonist-treated HSPC subset versus control results from corresponding HSPC subset that was untreated. Results are presented as the mean $\pm$ SEM. As stated in the

Introduction and shown in the Results section 3.1, 3.2, multiple external influences of inflammatory cytokines produced systemically, modify the behavior of HSPC in a systemic autoimmune inflammatory disease like lupus. Therefore, the responses of lupus HSPC to the apoptotic TLR agonists here cannot be directly compared to those in normal mice. Hence, data from lupus mice have their own controls and data from normal mice have their own controls; and they are presented as independent entities here. The Student two-tailed t tests were used for comparative analysis of untreated control HSPC subset versus apoptotic TLR agonist-treated HSPC subset within the normal mouse group, or within the lupus mouse group separately.

3. RESULTS

3.1. HSPC from normal and lupus mouse bone marrow produce IL-17 in response to apoptotic products and related TLR signals

3.1.1. Lupus prone mice have lower numbers of CD11b⁺ and CD3⁺ cells in bone marrow compared to normal mice—We used different isolation protocols for LSK cells or LSK-enriched cells in different experiments according to their aim, as mentioned below. As a *general approach*, bone marrow cells were harvested from cleaned bones after removal of any attached muscles, ligaments and lymph nodes and any peripheral blood contamination. LSK purification kits (Miltenyi Biotech) or direct FACS sorting did not yield functionally optimal cells for our assays, probably because supporting and interacting cells from the natural BM environment, such as BM stromal cells that support HSPC maintenance in the cultures, were eliminated. To obtain the large number of functionally competent LSK cells needed for the studies sections 3.1.-3.3., bead separation was used to obtain the LSK-enriched cells for cultures that lasted 1½ days, and then FACS gating was used to analyze the cultured cells to precisely identify and locate the cytokine producing HSPC in the harvested LSK cells *after* the 1½ day culture period. Thus, bone marrow cell fractionation was first done with magnetic beads to remove (and save) stepwise B220⁺ and CD11b⁺ cells, which make up the bulk of lymphoid population in marrow [38], and then CX3CR1⁺ cells [10, 39, 40], which contain Macrophage and DC progenitors (MDP), were removed. Finally, to obtain LSK-enriched cells, Sca-1⁺ cells were positively selected in the remaining population. The LSK-enriched cells here (sections 3.1.-3.3) included very few MKP and Megakaryocyte erythroid progenitors (MEP), as the latter are Sca-1⁻. In some experiments CD3⁺ cells were removed, but we eliminated this step, because there are very few CD3⁺ cells left after Sca-1⁺ cell selection, and the results of experiments in these sections were similar without a separate CD3 removal step; moreover, there is non-specific cell loss with each additional column step. Further selection to analyze cells of interest in these sections, such as CD117⁺ cells was done by gating in the flow cytometer *after* the LSK had been cultured with or without TLR agonists for 1½ days. Figure S1 shows gating strategy and steps for identifying HSPC subsets in cultured LSK cells (in the extreme right panel in lower row), as reported by others [41-43], and detailed below. It is notable in the FACS plots that small numbers of lineage⁺ (CD3⁺, B220⁺, and CD11b⁺) cells remained even after magnetic bead depletion, but they were then gated out to analyze the LSK cells (Figure S1) for HSPC markers (Lin⁻Sca-1⁺cKit⁺, and then by their CD34 vs. Flk2 expression). Indeed, the possibility of other IL-17 producing cells in the LSK-enriched

population differentiating in the 1½ day cultures does not detract from the findings and interpretation of results in this paper because, the HSPC were harvested and identified *after* the culture period by simultaneous expression of primitive stem and progenitor cell surface markers and intracellular IL-17 production in the same cell by flow cytometry, and not by IL-17 production assay in their culture supernatants.

Although total bone marrow cell yields, and B220⁺ cell and LSK cell frequencies were similar in lupus prone (NZB × NZW)F1 (B/WF1) and normal C57BL/6J (BL/6) mice, the B/WF1 mice had significantly lower numbers of CD11b⁺ and CD3⁺ cells in bone marrow ($p = 0.01$ and 0.005 respectively), compared to BL/6 mice. Data (mean ± SEM) from six independent experiments using a total of 44 mice showed the following. Average BM cell yield/mouse was $4.675 \pm 0.032 \times 10^7$ cells in the case of BL/6, and $4.76 \pm 0.66 \times 10^7$ in the case of B/WF1. The percentages of B220⁺, CD11b⁺, CD3⁺ and LSK cell frequencies among total bone marrow cells were 46.067 ± 3.083 , 41.1 ± 1.571 , 3.97 ± 0.423 and 0.346 ± 0.096 respectively in the case of BL/6, and 49.933 ± 3.624 , 30.833 ± 1.638 , 1.376 ± 0.205 and 0.415 ± 0.114 respectively in the case of B/WF1 mice.

3.1.2. HSPC from normal mice produce IL-17 upon stimulation by apoptosis related TLR agonists—Specifically for *each* experiment in these sections (3.1.-3.3) that deal with stimulation of HSPC with apoptotic TLR agonists, hematopoietic progenitor cells (LSK-enriched cells) from pooled bone marrow cells of ten 10-wk-old normal BL/6 female mice, or ten 10 week old female BWF1 lupus mice were isolated using magnetic bead conjugated anti-Sca-1 Ab after depletion of B220⁺, CD11b⁺ and CX3CR1⁺ cells. These LSK-enriched cells were cultured in triplicate with the TLR agonists, Poly (I:C) for TLR3; LPS for TLR4; R848 for TLR7/8 or CpG1585 for TLR9. In addition, the LSK cells were stimulated with the apoptotic products, namely, HMGB1-DNA complex, nucleosomes, necroptotic B cells, or apoptotic thymocytes, in a separate batch of animals, because the complex apoptotic products stimulate by multiple, known and unknown, mechanisms. IL-17 positive HSPC were detected by flow cytometry *after* 1½ days of culture. Each HSPC subset, namely LT-HSC, ST-HSC and MPP, in the cultured LSK cells was identified with the following phenotypes: LT-HSC, as Lin[−]CD117⁺Sca1⁺CD34[−]Flk2[−]; and ST-HSC being Lin[−]CD117⁺Sca1⁺CD34⁺Flk2[−]; and MPP, as Lin[−]CD117⁺Sca1⁺CD34^{low~}Flk2^{low~+} (CD117 = cKit; Flk2 = Flt3 or CD135) (Figures S1A). Only the CD34^{high+} cells based on FMO were considered here to be ST-HSC for further analysis of data. Indeed, this rigorous gating for ST-HSC was correct, as shown in Figure 2F below. The LT-HSC gate in Figure S1A, also contained other LSK cells (not MPP or ST-HSC), but this population produced the least amount of cytokines, as shown below. We also validated the above phenotypes with additional markers, such as, CD90.2^{low/intermediate+}CD150⁺CD38⁺CD48^{−/low+} for LT-HSC and ST-HSC, and CD90.2^{−/very low}CD150[−] for MPP, based on published work [39, 44-50]. The gating of HSPC subsets here was consistent with criteria established by many classical papers in the field that functionally validated such gated populations by in vivo repopulation studies and in vitro colony formation assays [39, 44-50]. Moreover, we confirmed the intracellular cytokine assay results further by using IL-17-GFP reporter mice and by intracellular staining for RORγt (shown below). It is notable that the MPP population in HSPC gated here also contain lymphoid primed multipotent progenitors (LMPP).

Importantly, the phenotype of the HSPC here was characterized *after* 1½ days of stimulation in culture, therefore expression of some of the markers, such as Flk2 is not as high as those found in freshly isolated MPP, nevertheless, the MPP were definitely positive for the marker. Figure 1F (and Figure S1 A) shows that the MPP have a fraction of cells that are CD34 negative or low⁺, as these cells were phenotyped after 1 ½ days of culture with stimulants. Nevertheless, they were gated for being Sca-1⁺CD117⁺; moreover, they were depleted of CX3CR1⁺ cells. Hence they could not be in the MDP (macrophage/dendritic cell progenitor) lineage. Thus, we examined IL-17 and IL-21 expression of the cultured HSPC after gating on their characteristic markers, such as being Lineage negative, c-Kit positive, Sca-1 positive and then based on their CD34 vs. Flk2 expression. Therefore, we conclude that those HSPC that still retained their primitive markers post-culture, can secrete IL-17 and IL-21 upon TLR ligand stimulation.

Representative flow cytometry plots of IL-17 production by the three subsets of HSPC from *normal* BL/6 mice in response to apoptotic products or related TLR ligands that stimulated well, are shown in Figure 1A, and FMO controls for gating are shown in Figures S2 **and** S3. Compiled results from 4 independent experiments for the TLR ligand group and the apoptotic product group are shown in Figure 1B and 1C respectively. The average yield of LSK cells per mouse was around 2×10^5 cells, consistent with published work [41]. Therefore, numbers of IL-17⁺ HSPC subsets per 2×10^5 LSK cells are shown in the *left panels* of Figure 1B (TLR ligands) and Figure 1C (apoptotic products), from experiments done with separate sets of animals. R848, CpG and HMGB-ssDNA complex stimulated the HSPC subsets to induce production of IL-17 significantly. Compared to control cultures, numbers of IL-17⁺ LT-HSC, ST-HSC and MPP increased markedly with R848 ($p = 0.0025$, 0.031 and 0.015 respectively); but with CpG and HMGB-DNA complex the increases were significant in LT-HSC and ST-HSC only ($p = 0.006$ and 0.034 with CpG, and $p = 0.027$ and 0.015 with HMGB-DNA, respectively). The same results are displayed as percentage of IL-17⁺ cells in each of the HSPC subsets, in the *right panels* of Figure 1B and 1C. Again R848, CpG and HMGB-ssDNA complex significantly increased frequency IL-17⁺ cells within each HSPC subset: % IL-17⁺ LT-HSC, ST-HSC and MPP increased markedly with R848 ($p = 0.002$, 0.026 and 0.002 respectively); but with CpG and HMGB-DNA complex the increases were significant in LT-HSC and ST-HSC only ($p = 0.009$ and 0.026 with CpG, and $p = 0.026$ and 0.046 with HMGB-DNA, respectively). MPP also showed a substantial increase with HMGB-DNA and CpG, but did not reach significance due to large variation. The marked increase in % IL-17⁺ MPP in response to the TLR 7/8 agonist R848 within the MPP subset (Figure 1B, *right panel*) suggests not only an increase in numbers of those cells but also relative depletion of IL-17 negative MPP cells either due to death or differentiation during the 1½ days of stimulation in culture. Similar situation might have occurred with some of the other subsets in response to HMGB-DNA stimulation.

The above results for intracellular IL-17 staining in HSPC by flow cytometry was confirmed by stimulating LSK cells from IL-17-GFP reporter mice ($n = 5$) with R848 and necroptotic B cells for 1½ days and then staining for HSPC surface markers and intracellular GFP expression in HSPC above controls. A representative histogram and compiled results are shown in Figure 1D. MPP in LSK cells stimulated by necrotic B or R848 showed significant

increases in GFP expression ($p = 0.033$ and 0.019 respectively), but any increase in ST-HSC and LT-HSC showed marked variation. Although confirmatory, the results were not as robust as intracellular IL-17 staining because of limitations of GFP as a reporter gene. GFP fluorescence is the final outcome of a complex process involving transcription, translation, and posttranslational modifications, which might not have occurred optimally in the HSPC in the short-term cultures, as it does in anti-CD3 stimulated CD4 T cell cultures under Th17 polarizing conditions (Jackson Laboratory catalog). Nevertheless, when LSK from C57BL/6 mice ($n = 5$) were cultured with the same stimulants followed by intracellular staining for ROR γ t, the transcription factor for IL-17, there was a strong positive response. An example with gated CD34 $^{+}$ ST-HSPC is shown in Figure 1E. During the 1½ day culture period some apoptotic products would be released from dying cells, which could have caused low ROR γ t expression in cultures without any deliberately added TLR agonists.

3.1.3. IL17 $^{+}$ HSPC results were not due to contamination by other IL-17 $^{+}$ cells

—We looked for other IL-17 producing cells in the bone marrow, such as ILC or T cells that could have contributed to the HSPC results. However, two of the gated populations, namely ST-HSC and MPP, which were the most significant IL-17/21 producers among the HSPC subsets have Flk2 and CD34 markers on their surface (Figure S1A), which are absent in ILC, and T cells, and in addition for excluding T cells, the HSPC were gated from CD3 negative cells (Figure S1). The same HSPC with those distinctive surface markers that are lacking in ILC and T cells, produced intracellular IL-17 and IL-21 (Figure 1F, *middle and lower rows*). Therefore, it is improbable for them to be ILCs, and those are the HSPC subsets (LSK cells with the distinct markers Flk2 and/or CD34 on surface and intracellular IL-17) that are shown in the results (Figures 1-5). Indeed, we did not measure IL-17 production by ELISA in the culture supernatants, because we aimed to detect precisely which HSPC was making IL-17, by staining for intracellularly produced IL-17 and distinct surface markers on the same individual cell in a HSPC subset, and those results are presented in Figures 1-5, and Figure 1F.

It is possible that some ILCs could be lurking in the lowest and least significant cytokine producing HSPC subset, the LT-HSC, because they are Flk2 and CD34 negative LSK cells, like LT-HSC. Indeed, the LT-HSC gate did contain other LSK cells (Figure S1A). However, that is unlikely for the following reasons: Among ILCs, NCR-ILC3 and LT α i produce IL-17 and express IL-23R. However LT α i cells are not LSK, because they express CD4 and are Sca-1 negative. Among the others, ILC1 cells are usually CD117 $^{-}$, and ILC3 cells are Sca-1 negative. That leaves us only with ILC2 cells that could be hiding in the LT-HSC subset, but they do not produce IL-17; they make Th2 cytokines (<http://www.ebioscience.com/knowledge-center/cell-type/innate-lymphoid-cells-ilcs>). Anyway, because HSC are CD90 $^{+}$ CD3 $^{-}$ CD150 $^{+}$, we had looked for IL-17 producing ILC among CD90 $^{+}$ CD3 $^{-}$ CD150 $^{-}$ cells in the LSK cultures after stimulating with IL-23 or apoptotic TLR ligands, but the results were negative.

It is also unlikely that any T cells that had downregulated CD3 after activation, could have masqueraded as IL-17 producing HSPC, because of the distinct surface markers of the latter cells as mentioned above. Moreover, as shown below in Section 3.5, Figure 7 B and C (shown later), IL-17 producing T cells are almost undetectable when LSK cells or bone

marrow T cells are cultured by themselves. Only when *co-cultures* of LSK and deliberately added T cells are stimulated by CpG or R848 TLR ligands, we see an increase in IL-17 producing T cells. Since CpG or R848 TLR ligands cannot stimulate T cells directly, it is the TLR-stimulated LSK cells that caused expansion of IL-17⁺ T cells, which are detectable only when T cells are deliberately added for co-culturing with the LSK cells.

Although, some of the HSPC could have differentiated during the cultures, we examined IL-17 and IL-21 expression of the cultured HSPC after gating on their characteristic primitive markers, such as being Lineage negative, c-Kit positive, Sca-1 positive and then based on their CD34 vs. Flk2 expression. Therefore, we conclude that those HSPC that still retained their primitive markers post-culture, can secrete IL-17 and IL-21 upon TLR ligand stimulation.

3.1.4. HSPC from lupus-prone mice were endogenously pre-stimulated to produce IL-17 and upon stimulation in vitro by apoptosis related TLR agonists their responses were much lower than normal HSPC—IL-17 production by the

three subsets of HSPC from *lupus* prone B/WF1 mice in response to known TLR ligands or apoptotic products, compiled from 2 separate experiments for TLR ligand group, and for apoptotic products group, are shown in Figure 2. Numbers of IL-17⁺ cells are shown in *left panels* of Figure 2A and 2B. The lupus HSPC subsets obtained from 10 wk old B/WF1 mice at pre-clinical stage of autoimmune disease, were spontaneously stimulated to produce IL-17 to begin with, as shown in the unstimulated “controls”. In the case of the TLR ligand group, when the lupus background or unstimulated controls are compared side by side with unstimulated background controls from normal mice (Figure 2C), the corresponding numbers (mean $\pm$ SEM) for LT-HSC were 1377.33 ± 265.77 and 140.66 ± 80.83 respectively ($p = 0.011$), and that for ST-HSC were 759.33 ± 131.39 and 32.67 ± 32.66 respectively ($p = 0.005$). However, in the case of MPP the corresponding numbers were 285.06 ± 73.15 in lupus controls, and 433.33 ± 86.66 in normal controls, indicating that this subset had been mobilized and/or differentiated in lupus. Nevertheless, further significant increases in the numbers of IL-17⁺ cells above the relatively high lupus background could be seen in the case of lupus ST-HSC with R848 ($p = 0.032$), LT-HSC with HMGB-DNA ($p = 0.05$), and MPP with LPS stimulation ($p = 0.047$). Regarding increase in percentages of IL-17⁺ HSPC within each subset (*right panels* of Figure 2), significant increases occurred in ST-HSC with R848 ($p = 0.004$) and CpG ($p = 0.05$), in LT-HSC with HMGB-DNA ($p = 0.05$), and in MPP with LPS ($p = 0.026$). MPP and ST-HSC were also stimulated well by Poly (I:C) and HMGB-DNA, but the increases in % IL-17⁺ cells did not reach significance due to high standard deviation. However, these increases in stimulated lupus HSPC were considerably lower than corresponding responses in normal mice when compared side by side ($p < 0.05$) (Figure 1B, C vs. Figure 2). It is notable that the Y-axis scales are different in the Figures showing results from the normal and lupus mice (Figure 1B, C vs. Figure 2), because of the lower responses in lupus HSPC. It is problematic to display all of the ICS results from normal and lupus mice in one figure, because lupus HSPC responds much less than normal HSPC, and therefore lupus data would be miniaturized and obscured when cluttered together with those from normal mice.

3.2. HSPC from normal and lupus bone marrow also produce IL-21 in response to apoptotic products and related TLR signals

Again for *each* of these experiments, bone marrow hematopoietic progenitor cells (LSK-enriched cells) pooled from ten 10-wk-old normal BL/6 female mice or ten 10 week old female BWF1 lupus mice were isolated as in the above section and then IL-21 responses to apoptotic products and related TLR agonists were assayed after 1½ days of stimulation in culture.

3.2.1. HSPC from normal mice produce IL-21 upon stimulation by apoptosis related TLR agonists—Representative flow cytometry plots of IL-21 production by the HSPC subsets from *normal* BL/6 mice in response to apoptotic products or related TLR ligands that stimulated well, are shown in Figure 3A, and FMO controls for gating are shown in Figures S2 and S3. Compiled results from 4 independent experiments are shown in Figure 3B and C. Numbers of IL-21⁺ HSPC subsets in 2×10⁵ LSK cells are shown in the *left panels* of Figure 3B (TLR ligands group) and Figure 3C (apoptotic products group), from experiments done with separate sets of animals. R848, CpG and HMGB-ssDNA complex stimulated the HSPC subsets to induce production of IL-21 significantly. Compared to control cultures, numbers of IL-21⁺ LT-HSC, ST-HSC and MPP increased markedly with R848 ($p = 0.00002$, 0.05 and 0.0008 respectively), but with HMGB-DNA complex the increases were significant in LT-HSC and ST-HSC only ($p = 0.047$ and 0.0003, respectively). In addition, there were modest but significant increases of numbers of IL-21⁺ cells in LT-HSC upon stimulation by necroptotic B cells and apoptotic thymocytes ($p = 0.02$ and 0.04 respectively).

The same results are displayed as percentage of IL-21⁺ cells in each of the HSPC subsets, in the *right panels* of Figure 3B and 3C. Again R848, CpG and HMGB-ssDNA complex significantly increased frequency IL-21⁺ cells within each HSPC subset: % IL-21⁺ LT-HSC, ST-HSC and MPP increased markedly with R848 ($p = 0.00002$, 0.01 and 0.000000003 respectively), and with CpG ($p = 0.03$, 0.001 and 0.007, respectively). With HMGB-DNA complex stimulation, the increases were significant in LT-HSC and ST-HSC only ($p = 0.046$ and 0.000006, respectively). Again, MPP also showed a substantial increase, but did not reach significance due to large variation. Similar to IL-17, the marked increase in % IL-21⁺ MPP in response to the TLR 7/8 agonist R848 within the MPP subset suggests not only increase in numbers of those cells but also relative depletion of IL-21 negative MPP cells either due to death or differentiation during the 1½ days of stimulation in culture. Similar situation might have also occurred with some of subsets in response to HMGB-DNA stimulation. In addition to above there were modest but significant increases of % IL-21⁺ cells in LT-HSC in response to stimulation by necroptotic B cells and apoptotic thymocytes ($p = 0.006$ and 0.04 respectively).

3.2.2. HSPC from lupus-prone mice were endogenously pre-stimulated to produce IL-21 and upon stimulation in vitro by apoptosis related TLR agonists their responses were much lower than normal HSPC—IL-21 production by the HSPC subsets from 10 wk old *lupus* prone B/WF1 mice in response to apoptotic products or related TLR ligands, compiled from 2 separate experiments for each group (TLR ligands or

apoptotic products) are shown in Figure 4. Numbers of IL-21⁺ cells are shown in *left panels* of the Figure 4A and B. Again, the lupus HSPC subsets were spontaneously (endogenously) stimulated to produce IL-21 to begin with, as shown in the “controls”. In the TLR ligand stimulation group, when the lupus background controls are compared side by side with unstimulated background controls from normal mice (Figure 4C), the corresponding numbers (mean $\pm$ SEM) for LT-HSC were 1238 ± 336.09 and 586.67 ± 159.3 respectively, and that for ST-HSC were 1269.33 ± 327.86 and 80 ± 40.26 ($p = 0.02$) respectively. However, in the case of MPP the trend was reversed, being 23.6 ± 23.15 in lupus controls, and 199.33 ± 42.27 in normal controls ($p = 0.022$), indicating that this subset had been mobilized and/or differentiated in lupus. Nonetheless, further significant increases in the numbers of IL-21⁺ cells above the relatively high lupus background could be seen in the case of MPP with R848 stimulation ($p = 0.023$); LT-HSC and ST-HSC with HMGB-DNA ($p = 0.016$ and 0.027 , respectively); and low but significant increases in LT-HSC and MPP with necroptotic B cells ($p = 0.014$ and 0.008 , respectively). Regarding increases in percentages of IL-21⁺ HSPC within each subset (*right panels* of Figure 4), significant increases occurred in MPP with R848 ($p = 0.023$); in LT-HSC and ST-HSC with HMGB-DNA ($p = 0.001$ and 0.012 , respectively); and low but significant increases in LT-HSC and MPP with necroptotic B cells ($p = 0.037$ and 0.025). MPP were also stimulated well by Poly (I:C), LPS, and HMGB-DNA, but the increases in IL-21⁺ cells did not reach significance due to high standard deviation. However, these increases in stimulated lupus HSPC were considerably lower than corresponding responses in normal mice when compared side by side. For increases in numbers of IL-21⁺ HSPC responding in R848 stimulated MPP, normal vs. lupus $p = 0.0000081$; that for necrotic B cell stimulated LT-HSC, $p = 0.0016$, and HMGB-DNA stimulated ST-HSC, $p = 0.00029$. For increases in % of IL-21⁺ HSPC responding in R848 stimulated MPP, normal vs. lupus $p = 0.0000044$ (Figure 3B,C vs. Figure 4).

3.3. MPP among HSPC show enhanced cycling in response to apoptotic products and related TLR signal

LSK enriched cells from BL/6 mice ($n=5$) were isolated according to the protocol in above sections and then cultured for 1½ days with the TLR7/8 agonist R848, the best stimulant for IL-17 and IL-21 induction in HSPC (Figure 1 and 3). Cell cycle status in the stimulated HSPC subsets were analyzed by Ki-67 and DAPI staining, and percentages of cells in each HSPC subset in quiescent and resting (G_0/G_1) and cycling ($S-G_2/M$) state were determined according to published methods [37, 51]. Figure 5 shows that most of the LT-HSC and ST-HSC remained in resting state even after stimulation, whereas a substantial proportion of MPP were in cycling state ($S-G_2/M$ or DNA synthesis/mitosis and replication state) with R848 stimulation. The 1½ day cultured cells showed low level cycling without stimulation.

3.4. Hematopoietic stem and progenitor cells express mRNA for many other cytokines for augmenting and maintaining memory T cells in normal and lupus bone marrow

The HSPC were capable of producing other cytokines besides IL-17 and IL-21. LSK cells were prepared from seven BL/6 and twelve B/Wf1 mice side by side using a variation of the protocol in above sections with the following order of separation by microbeads: B220⁺, CD11b⁺, CD3⁺, CX3CR1⁺, and CD41⁺ cells were removed (and saved) followed by

positive selection of CD117⁺ cells. The LSK-enriched cells devoid of T cells and MKP and MEP (MM cells) were then cultured with R848 or CpG1585 for 16 hours followed by RNA extraction for qPCR in triplicates. RNA from B220⁺ cells from bone marrow were used as a background control. Figure 6 shows that cytokine mRNA were expressed in low levels by the LSK-enriched cells without addition of the TLR agonists, but upon stimulation, the LSK enriched cells from both normal and lupus mice expressed high levels of mRNA for cytokines required for inducing and expanding Th17 cells, such as IL-6, IL-1, IL-18, TNF α [52-57]. Although IL-23 α mRNA was modestly increased, its partner IL-12 β , required for the whole IL-23 molecule [58], was highly expressed. Moreover, the LSK enriched cells expressed mRNA for other mediators that have been implicated in Th17 induction, namely BAFF, and COX2 (PTGS2) the latter mediating PGE2 production [59, 60]. TGF β mRNA was not increased above the controls (not shown), probably because it is activated from preformed latent precursor (LAP) already present in the cells. The LSK cells also expressed mRNA for IL-7 and IL-15 required for memory T cell maintenance, although stromal cells in the bone marrow were previously thought to be the only source of these cytokines [61-64]. The LSK cells did not express IL-2 mRNA (not shown). The high level expression of GM-CSF mRNA by HSPC is probably a downstream effect of IL-17 production [65] by the HSPC themselves, which may have acted in an autocrine/paracrine fashion. These cytokines and mediators induced by the TLR agonists also have effects on other cells in the bone marrow, as discussed later.

3.5. Hematopoietic stem and progenitor cells stimulated by apoptotic products and related TLR signals augment memory Th17 and Tc17 cells in normal and lupus bone marrow

In these experiments bone marrow T cells and LSK cells were co-cultured in presence of the stimulants to study effects on memory T cells. Bone marrow cell fractionation was modified for this purpose. In the first set of experiments below, B220⁺ cells, CD11b⁺ cells, CD90⁺ cells, CX3CR1⁺ cells and CD41⁺ cells were removed (and saved) stepwise and then CD117⁺ cells were positively selected in the remaining population to yield LSK enriched (LSKen) cells. Sca-1⁺ cells were further selected in an aliquot of the LSK-enriched cells to yield pure LSK cells, but there were no remaining Sca-1⁻ (LS⁻K) cells left after this magnetic bead isolation steps, showing that LSKen cells here are almost entirely pure LSK population, which is consistent with functional studies shown below. The LSKen and LSK cells here do not contain MKP and MEP (MM cells), because the former (LSK) cells are Sca-1⁺; moreover, CD41⁺ cells were removed during their purification. Next, the isolated and saved CD90⁺ cell fraction that contained bone marrow T cells, LT-HSC and some ST-HSC, were mixed with the LSKen or LSK cells that mainly contained MPP, LMPP and some ST-HSC, for co-culture in triplicate in the presence of apoptotic products and related TLR stimulants for 1½ days, and then IL-17⁺ naïve and memory T cells were analyzed in CD3-gated CD4 and CD8 T cell populations by flow cytometry.

Figure 7A shows gating strategy for CD4 and CD8 naïve and memory T cell subsets, based on CD44 and CD62L. Naïve CD4 T cells are CD62L^{high}CD44^{-low}CD69⁻, central memory CD4 T cells (CD4 T_{CM}) are CD62L⁺CD44^{high}CD69⁻, and effector memory CD4 T cells (CD4 T_{EM}) are CD62L⁻CD44^{high}CD69⁺, as published [66]. The CD8 memory T cells are divided further into CD8 T_{CM} (CD62L⁺CD44^{high}CD69⁻), CD8 T_{EM}

(CD62L⁻CD44^{high}CD69⁺), and CD8 T stem cell memory or CD8 T_{SCM} that are CD62L⁺CD44^{low/intermediate}CD69⁻, as published [67-69]. All these subsets were found in the bone marrow of BL/6 and B/WF1 mice. An example of unstimulated cells from bone marrow of normal BL/6 mice is shown in Figure 7A.

Figure 7B and C show results from *normal* BL/6 mice (n = 12), the frequency of IL-17⁺ memory T cells in CD90⁺ bone marrow cells co-cultured in triplicate with LSKen or LSK cells in the presence of R848 (TLR7/8 agonist), CpG (TLR9 agonist), nucleosomes and necroptotic B cells, and controls without the stimulants. It is notable that IL-17 producing T cells were almost undetectable when LSK cells, or bone marrow T cells are cultured by themselves. Only when *co-cultures* of LSK and deliberately added T cells are stimulated by CpG or R848 TLR ligands, we see an increase in IL-17 producing T cells. Since CpG or R848 TLR ligands cannot stimulate T cells directly, it is the TLR-stimulated LSK cells that caused expansion of IL-17⁺ T cells, which were detectable only when T cells are deliberately added for co-culturing with the LSK cells. Thus, R848 and CpG stimulation significantly increased the % of IL-17⁺ T cells in all subsets in the co-cultures. R848 increased IL-17⁺ “naïve” CD4 T with either LSKen or with LSK co-cultures ($p = 0.0003$ and 0.00004 respectively), IL-17⁺ CD4 T_{CM} ($p = 0.0002$ and 0.000002 respectively) and IL-17⁺ CD4 T_{EM} ($p = 0.045$ and 0.028 respectively), shown in Figure 7B. Thus the stimulated LSKen and LSK cells were equivalent in providing the cytokine/mediators/signals for IL-17⁺ T cell expansion, and the T cells did not manifest this expansion when cultured by themselves with the stimulants. It is notable that the “naïve” IL-17⁺ T cells expanded in 1½ days of co-culture with stimulated LSK, without any polarizing conditions, indicating that these are pre-primed IL-17⁺ T cells in the bone marrow. CpG also increased IL-17⁺ “naïve” CD4 T and IL-17⁺ CD4 T_{CM} with LSKen co-cultures ($p = 0.005$ and 0.002 respectively). Co-cultures with LSK were not done with CpG. In the case of CD8 T cells (Figure 7C), R848 increased IL-17⁺ CD8 T_{CM} and CD8 T_{SCM} with LSKen or LSK co-cultures ($p = 0.002$ and 0.007 respectively), and IL-17⁺ CD8 T_{EM} increased significantly with LSKen co-culture ($p = 0.01$). CpG stimulation increased IL-17⁺ CD8 T_{CM} and T_{SCM} with LSKen co-cultures ($p = 0.005$) and IL-17⁺ CD8 T_{EM} also increased significantly with the LSKen co-culture ($p = 0.0001$). LSK co-cultures were not done with CpG.

Figure 8, shows results from lupus prone B/WF1 mice. In the experiments above (Figure 7B,C), there were no responses to nucleosomes or necroptotic B cells, probably because CD41⁺ cells were removed from the LSKen or LSK cells preparation, and because the frequency of autoimmune T cells that can respond to these autoantigens is vanishingly rare in normal mice [10, 23]. The CD41⁺ CD117⁺ cells contain MKP and MEP (MM cells), which can process and present autoantigens from apoptotic particles like nucleosomes. In contrast to HSPC, MKP do not produce IL-17 when presented with apoptotic TLR agonists, but they induce and expand Th17 in lupus by processing and presenting apoptotic nucleosome particles [10, 23]. HSPC, unlike MKP do not have phagocytic ability or APC function [23, 29]; they augmented memory T cells in a non-cognate or non-antigen specific manner by the cytokines and mediators they produced on stimulation of their TLRs (Figure 7B,C). Therefore, we modified the LSKen cell isolation protocol in this set of experiments to include CD41⁺ (MM) cells, and used T cells from lupus mice as responders. Thus from

the bone marrow cells, B220⁺ cells, CD11b⁺ cells, CD90⁺ cells, and CX3CR1⁺ cells were removed (and saved) stepwise and then CD117⁺ cells were positively selected in the remaining population containing both Sca-1⁺ and Sca-1⁻ cells, to yield LSK enriched cells that also contained the MM cells (LSKen & MM). Because the frequency of autoantigen specific T cells are low, the T cell responses to particulate autoantigens from necrotic B cells or nucleosomes processed and presented by MM cells in the mixture (LSKen & MM) were modest as compared to non-specific memory T cell expansion by TLR-ligand stimulated HSPC (Figure 7B,C), but they were clearly significant when lupus bone marrow CD90⁺ cells were co-cultured in triplicate with LSKen & MM (Figure 8). With necroptotic B cells in culture, increases in percentages of IL-17⁺ Naïve CD4 T, CD4 T_{CM} and CD4 T_{EM} were evident ($p = 0.001$, 0.0004 and 0.048 respectively) and with nucleosomes increases occurred in IL-17⁺ Naïve CD4 T and CD4 T_{CM} ($p = 0.013$, and 0.026 respectively), shown in Figure 8A. In the case of CD8 T cells (Figure 8B) increases of IL-17⁺ CD8 T_{CM} and T_{SCM} occurred in response to necrotic B cells and nucleosomes ($p = 0.01$ and 0.028 respectively). We had previously found that MKP and MEP (MM) cells are expanded in the periphery of lupus prone mice and lupus patients, as a part of extramedullary hematopoiesis, and they act as professional APC to induce and expand nucleosome-specific Th17 cells [10]. Therefore, we tested here whether purified *bone marrow* MKP and MEP (MM) cells behaved similarly to their peripheral counterparts [10, 23], in their ability to process and present nucleosomes to induce and augment autoimmune Th17 response in ELISPOT assays. Bone marrow MM cells were further purified from bead fractionated LSK cells by cell sorting as a CD41⁺CD117⁺ population. These bone marrow MM were more efficient than other APC from bone marrow, such as CD11b⁺ cells, or CX3CR1⁺ macrophage/DC progenitors; or APC from spleen, such as, splenic CD11c⁺ cells or splenic MM cells of B/WF1 mice, in processing and presenting nucleosome particles to augment nucleosome specific autoimmune Th17 response in splenic T cells of B/WF1 lupus mice (Figure 8C) even at a 1:42 ratio of MM cells to T cells, thus supporting the results in Figure 8A and B.

4. DISCUSSION

4.1. Herein, we show that HSPC themselves are induced to produce IL-17, IL-21 and many other cytokines/mediators upon stimulation by *endogenous* TLR agonists associated with apoptotic cell products that are a part of ongoing developmental process in the hematopoietic environment. We also found that one aspect of the functional consequence of these stimulated, cytokine producing HSPC was sustenance and expansion of IL-17 producing memory T cells in the bone marrow. The strongest and consistent inducers of cytokine production by HSPC were HMGB1 complexed with DNA, and R848, a TLR 7/8 agonist. HMGB1 is a nuclear protein, which is released from damaged cells and provides a danger signal (DAMP) through several receptors, such as TLR4 and RAGE to activate innate immune cells in the host [70]. HMGB1 by itself is relatively inert, but when complexed with DNA or nucleosomes, as released from late apoptotic cells, it becomes highly inflammatory and contributes to the pathogenesis of lupus [7, 11]. The other strong stimulator of HSPC was the TLR 7/8 ligand R848, a surrogate for endogenous ribonucleoprotein RNA found in apoptotic cell products. Again, exaggerated TLR 7/8 stimulation contributes to lupus by activating various cells of the immune system, such as

APC and B cells [16-19]. Herein, both of these TLR agonists stimulated HSPC of normal mice very strongly to produce the cytokines and go into cell cycle. The TLR 9 agonist CpG also stimulated the HSPC, but variably so. In the lupus bone marrow, we found that the HSPC were already pre-stimulated endogenously to produce the cytokines spontaneously, but some TLR agonists stimulated them further. These results are consistent with findings of abnormal HSC mobilization in bone marrow of lupus mice [71, 72], and suggests that endogenous TLR ligands from apoptotic products, whose clearance is defective in lupus, might be playing a role in stimulating the HSPC. The pre-stimulated lupus HSPC would be exhausted and desensitized to further stimulation by apoptotic TLR agonists. Moreover, some of the lupus HSPC might have differentiated and/or migrated out of the bone marrow to sites of extramedullary hematopoiesis, due to the influence of inflammatory cytokines, such as IL-1, IL-17, GM-CSF or type I interferons produced elsewhere systemically in lupus [10, 23]. For these reasons, the responses of lupus HSPC appeared to be less robust compared to normal HSPC (Figure 1 vs. Figure 2, and Figure 3 vs. Figure 4; **Results section (3.1.4 and 3.2.2)**). It is probable that other IL-17⁺ cells, such as ILC, or activated T cells that had downregulated CD3 could have contaminated and differentiated in the 1½ day LSK cultures. However, that possibility does not detract from the findings and interpretation of results in this paper because, the HSPC were harvested and identified *after* the culture period by simultaneous expression of primitive stem and progenitor cell surface markers and intracellular IL-17 production in the same cell by flow cytometry. Moreover, we did not find the presence of IL17⁺ ILC in LSK after the cultures (Results Section **3.1.3**).

4.2. Possible functional consequences of cytokine production by the stimulated HSPC are numerous. Studies with exogenously added IL-17 has shown that it can induce bone marrow stromal cells and mesenchymal stem cells to proliferate and produce inflammatory and hematopoietic cytokines [73-76]. Exogenous IL-17 from Th cells has also been shown to mobilize HSPC and augment their hematopoietic activity [77]. By contrast, another study showed T cell derived IL-21 but not IL-17 was responsible for HSPC mobilization and repopulating activity [78]. Thus, IL-17 and IL-21 produced by the HSPC in response to endogenous TLR agonists could act in an autocrine or paracrine manner to mobilize the HSPC. However, we found that addition of an antibody to IL-17 did not affect cell cycle status of HSPC stimulated by endogenous TLR ligands in culture (data not shown).

The stimulated HSPC expressed mRNA for several cytokines for sustaining memory Th17 cells in the bone marrow such as IL-6, IL-1, IL-18, TNFα [52-57] as well as BAFF and COX2 (PTGS2) the latter mediating PGE2 production [59, 60]. IL-21, secreted by the HSPC could have multiple effects, as it is known to augment RORγt and IL-23 R expression, thus maintaining and promoting Th17 memory cells; it is also required for Tfh cell differentiation by upregulating expression of BCL-6 and MAF, and is well known for its critical role in B cell differentiation and germinal center formation [79]. The stimulated HSPC also expressed mRNA for IL-7 and IL-15 required for memory T cell maintenance. Previously stromal cells in the bone marrow were thought to be the source of these cytokines [61-64]. The high level expression of GM-CSF mRNA is probably a downstream effect of IL-17 production [65] by the HSPC themselves, which may have acted in an autocrine/paracrine fashion. These cytokines and mediators produced by the HSPC upon stimulation by apoptotic TLR agonists

also have effects on other cells in the bone marrow. IL-6, IL-21, BAFF would stimulate or sustain B cells and long lived plasma cells, and they are also produced by MKP [10, 23], and IL-6 by mature megakaryocytes [80]. Furthermore, GM-CSF produced by the HSPC is well known for proliferation and mobilization of hematopoietic progenitors.

4.3. The HSPC, stimulated by the apoptotic TLR agonists, caused expansion of all subsets of IL-17 producing memory CD4 and CD8 T cells, namely CD4 T_{CM} and CD4T_{EM}; and CD8 T_{CM}, CD8 T_{SCM} and CD8 T_{EM}. Notably, IL-17 producing T cells with naïve phenotype also expanded in 1½ days of co-culture with the stimulated HSPC, without any polarizing conditions, indicating that these are pre-primed, antigen-experienced IL-17⁺ T cells in the bone marrow, and that stimulated HSPC provide not only cytokines but other uncharacterized mediators/signals for their rapid expansion. In contrast to HSPC, MKP in the bone marrow did not produce IL-17 when presented with apoptotic cell products, but they induced Th17 in lupus strains by processing and presenting apoptotic nucleosomes. HSPC, unlike MKP do not have APC function to process nucleosomes from apoptotic cells, although they are stimulated directly by TLR agonists. MKP take up and process antigens efficiently; they have the endosome/lysosome machinery of professional APC, expressing mRNA for Lyz, IRF5 and Cathepsins, and they express HLA-DR highly, and produce all cytokines necessary for inducing and expanding Th17 and Th1/Th17 potentially pathogenic T cells [10, 23]. Remarkably, MHC class II expression is lost as the MKP mature and they also lose the capacity to present nuclear autoantigens to Th cells with maturation [10, 23]. Indeed, matured platelets unlike MKP, are limited by their expression of only MHC class I, like stromal cells, and they do not augment Th17 responses [81-83]. Herein, we found that MKP and MEP (MM) cells in the bone marrow behaved similarly to their peripheral counterparts [10, 23], in their ability to process and present nucleosomes to induce and augment autoimmune Th17 response. Remarkably, the stimulated HSPC did not augment Th1 cells (data not shown); probably because CX3CR1⁺ MDP and myeloid progenitors, which have Th1 augmenting ability [10], were removed during HSPC purification here. While this work was in progress several observations had indicated that primitive hematopoietic progenitor cells are capable of producing cytokines in response to exogenous TLR 4 or TLR 2 agonists, such as LPS or PamCys [23, 84], but functional consequences of such observations were not studied. Herein, we show the effect of endogenous TLR ligands generated by ongoing developmental process in the hematopoietic environment on HSPC and one aspect of their functional consequence is production of cytokines by the primitive HSPC for sustenance and expansion of memory T cells (IL-17⁺) in the bone marrow. Thus the apoptotic TLR stimulated HSPC are both directly and indirectly responsible for contributing to the inflammatory milieu inside the bone marrow.

4.4. Concluding Remarks

Thus the earliest hematopoietic stem and progenitor cells in the bone marrow, stimulated by apoptotic cell product related endogenous TLR agonists, can produce cytokines/mediators to sustain and augment memory T cells in the bone marrow, and reciprocally cytokines from the stimulated T cells can augment hematopoiesis and HSPC mobilization. The findings in this study also have implications for B cells in the marrow. The cytokines/mediators produced by HSPC, MKP and memory T cell interactions could influence developing B

cells, which constitutively have a large repertoire cross-reactive with nuclear autoantigens even in normal subjects [85-88]. Moreover, autoimmune memory T cells reside and traffic through the bone marrow [89, 90]. Therefore, tolerance defects in the developing B cell compartment in lupus may not be exclusively B-cell intrinsic. HSPC/MKP and memory T cell interactions stimulated by apoptotic TLR agonists in the bone marrow might contribute to tolerance breakdown in lupus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Normal marrow HSPC produce IL17/21 & other cytokines on apoptosis related TLR agonist stimulation
- Apoptotic product/TLR stimulated normal HSPC augment IL-17⁺ memory CD4 & CD8T cells in bone marrow
- Lupus bone marrow HSPC are endogenously pre-stimulated to produce IL17/IL 21 and other cytokines.
- In contrast to bone marrow HSPC, megakaryocyte progenitor cells (MKP) do not produce IL-17/IL 21.
- Unlike HSPC, MKP can process & present apoptotic antigens, augmenting autoimmune Th17 in lupus.

Figure 1.

IL-17 production by HSPC from normal mice in response to apoptotic products and related TLR ligands. (A) Representative flow cytometry plots of HSPC for IL-17 production. Lineage⁻Sca-1⁺cKit⁺ (LSK) enriched cells from normal BL/6 mice were cultured with apoptotic products (HMGB1-ssDNA complex and necrotic B cells) or related TLR ligands (CpG1585 and R848) for 1½ days. Three subsets of HSPC, LT-HSC, ST-HSC and MPP, were identified in the cultured LSK cells, for their expression of CD34 and Flk2, as described in Figure S1A. Intracellular IL-17 expression of each HSPC subset after

stimulation is shown here. The IL-17 positive gating was determined based on fluorescence-minus-one (FMO) and biological control samples (Figures S2 and S3). Compiled results of IL-17 responses are shown in **(B)** and **(C)**. LSK enriched cells of normal mice were cultured with TLR ligands **(B)** and apoptotic products **(C)** for 1½ days, and IL-17 production was detected by flow cytometry. The IL-17 positive gating of each HSPC subset was determined based on fluorescence-minus-one (FMO) and biological control samples. Numbers of IL-17⁺ HSPC subsets in 2×10⁵ LSK cells are shown in the left panels and frequencies (%) of IL-17⁺ HSPC subsets are shown in the right panels. Data (mean ± SEM) are from 4 independent experiments using 40 mice per group, **(B)** and **(C)**. **(B)** Among TLR ligands, R848 stimulation significantly increased both numbers and percentages of IL-17⁺ LT-HSC, ST-HSC and MPP. Whereas, CpG1585 significantly increased numbers and percentages of IL-17⁺ LT-HSC and ST-HSC. **(C)** Among apoptotic cell products, HMGB-DNA complex stimulated increases in IL-17⁺ cell numbers and percentages significantly in LT-HSC and ST-HSC only. Although MPP also showed a substantial increase in response to apoptotic product stimulation, it did not reach significance due to large variation. In this and subsequent figures, significant *p* values are designated by *, ** or *** (*P* < 0.05, < 0.01 and < 0.001 respectively), and given in detail in Results section 3.1. Next, to confirm the intracellular IL-17 response of HSPC in response to apoptotic products stimulation, LSK enriched (LSKen) cells from IL-17-GFP reporter mice (C57BL/6-II17a^{tm1Bcgen}/J, *n* = 5) were cultured with R848 and necrotic B cells for 1½ days and then stained for HSPC surface markers and intracellular GFP expression in HSPC. Representative example and compiled results are shown in **(D)**: GFP fluorescence in MPP stimulated by necrotic B cell (open histogram) significantly increased as compared to unstimulated MPP (shaded histogram). **(E)** The IL-17 responses in stimulated HSPC were also confirmed by the expression of RORγt. LSKen cells from C57BL/6 mice (*n* = 5) were cultured with R848 and necrotic B cells for 1½ days and then intracellularly stained for RORγt expression. A representative result is shown: RORγt expression in R848 stimulated ST-HSC (open histogram) increased compared to unstimulated ST-HSC (shaded histogram). Negative control (FMO of total LSK cells) is shown by the curve with the dotted line. **(F)** Representative plots of HSPC in LSK cells that had been cultured with R848. The *top row* shows three subsets of HSPC, LT-HSC, ST-HSC and MPP that were identified in the cultured LSK cells, for their expression of CD34 and Flk2, as described in Figure S1, but from a different BL/6 animal here. Intracellular IL-17 production in those gated LT-HSC, ST-HSC and MPP cells are in this row (shown as CD117 vs. IL-17). The *middle row* shows cells in the HSPC subsets that were dually positive for CD34 and intracellular IL-17 in the same cell. The *bottom row* shows the cells in HSPC subsets that were dually positive for Flk2 and intracellular IL-17 in the same cell.

Figure 2.

HSPC from lupus mice, were endogenously pre-stimulated for IL-17 production, but could be further stimulated modestly by certain apoptotic cell products and related TLR ligands. LSK enriched cells from 6-10 wk old lupus prone mice at pre-clinical disease stage, were cultured with apoptosis related TLR ligands (A), and apoptotic products (B), for 1½ days, and IL-17 production was detected by flow cytometry. Numbers of IL-17⁺ HSPC subsets in 2x10⁵ LSK cells are shown in the left panels and frequencies (%) of IL-17⁺ HSPC subsets are shown in the right panels. Data (mean ± SEM) represent two independent experiments

using 22 mice per group. The lupus HSPC subsets were endogenously stimulated to produce IL-17 spontaneously (as shown by the 'controls' in **A** and **B**). Further increases in the numbers of IL-17⁺ cells significantly above the high background were observed upon stimulation with certain apoptotic products and related TLR ligands, such as ST-HSC with R848, LT-HSC with HMGB-DNA, and MPP with LPS stimulation (**A** and **B**, *left panels*). The percentages of IL-17⁺ cells were significantly increased with the following stimulants: ST-HSC with R848 and CpG, LT-HSC with HMGB-DNA, and MPP with LPS (**A** and **B**, *right panels*). The percentages of IL-17⁺ MPP with Poly (I:C) stimulation and IL-17⁺ ST-HSC with HMGB-DNA stimulation were also increased. However, the increases did not reach significance due to high variation. Significant *p* values are shown, as in Figure 1 B,C, and given in detail in Results section **3.1. (C)**. For comparison, results of normal mouse control group (unstimulated) next to the lupus mouse control group (unstimulated) are shown side-by-side using same Y-axis scale.

Figure 3.

HSPC from normal mice produced IL-21 in response to apoptotic products and related TLR ligand stimulation. (A). Representative flow cytometry plots of IL-21 production by stimulated HSPC. LSK enriched cells from normal BL/6 mice were cultured with apoptotic products (HMGB1-ssDNA complex and necrotic B cells) or related TLR ligands (CpG1585 and R848) for 1½ days. Three subsets of HSPC, LT-HSC, ST-HSC and MPP, were identified by the expression of CD34 and Flk2 (as in Figure S1). The IL-21 production of each HSPC subset after stimulation is shown. The IL-21 positive gating of each HSPC subset was determined based on fluorescence-minus-one (FMO) and biological control samples (Figures S2 and S3). Compiled results of IL-21 responses are shown in **Figure 3B** and **C**. LSK enriched cells were cultured with TLR ligands (B), and apoptotic products (C), for 1½ days, and IL-21 production was detected by flow cytometry. Numbers of IL-21⁺ HSPC subsets in 2x10⁵ LSK cells are shown in the left panels and frequencies of IL-21⁺ HSPC subsets are shown in the right panels. Data (mean ± SEM) represent 4 independent experiments using 40 mice. (B). R848 stimulation increased the numbers of IL-21⁺ LT-HSC, ST-HSC and MPP and the percentages of IL-21⁺ LT-HSC, ST-HSC and MPP markedly. CpG1585 significantly increased the percentages of IL-21⁺ LT-HSC, ST-HSC and MPP. (C). With HMGB-DNA complex stimulation, the increases of numbers and

percentages of IL-21⁺ cells were significant in LT-HSC and ST-HSC only. MPP showed increased numbers and percentage of IL-21⁺ cells with HMGB-DNA stimulation, however, the increases were not significant due to large standard deviation. In addition, there were modest but significant increases in numbers and percentages of IL-21⁺ cells in LT-HSC upon stimulation by necrotic B cells and apoptotic thymocytes. Significant *p* values are shown, as in Figure 1B,C, and given in detail Results section **3.2**.

Figure 4.

IL-21 production by lupus HSPC in response to apoptotic products and related TLR ligands. LSK enriched cells from 6-10 wk old lupus prone B/Wf1 mice at pre-clinical disease stage were cultured with apoptosis related TLR ligands (A), and apoptotic products (B), for 1½ days, and IL-21 production was detected by flow cytometry. Numbers of IL-21⁺ HSPC subsets in 2x10⁵ LSK cells are shown in the left panels and frequencies of IL-21⁺ HSPC subsets are shown in the right panels. Data (mean and SEM) represent two independent experiments with 22 mice. Lupus HSPC were endogenously pre-stimulated and producing

IL-21 without any further stimulation (**A & B**, 'controls'). (**A**). IL-21 producing MPP cell numbers and percentage could be further increased with R848 stimulation. (**B**). HMGB-DNA increased both numbers and frequencies of IL-21 producing LT-HSC and ST-HSC; and low but significant increases in LT-HSC and MPP occurred with necrotic B cells. MPP were also stimulated well by Poly (I:C), LPS, and HMGB-DNA, but the increases in IL-21⁺ cells did not reach significance due to high standard deviation. Significant *p* values are shown, as in Figure 1B,C, and given in detail in Results section **3.2**. (**C**). For comparison, results of normal mouse control group (unstimulated) next to the lupus mouse control group (unstimulated) are shown side-by-side using same Y-axis scale.

Figure 5.

Apoptotic cell products promoted MPP to enter cell cycling state. LSK enriched cells from BL/6 mice (n=5) were cultured for 1½ days with the TLR7/8 agonist R848. Cell cycle status in the stimulated HSPC subsets was analyzed by Ki-67 and DAPI staining. **(A)**. After stimulation, most of the LT-HSC and ST-HSC remained in quiescent and resting state (G₀/G₁). A substantial proportion of MPP were in cycling state (S-G₂/M or DNA synthesis/mitosis and replication state) with R848 stimulation, compared to the control group which showed low level cycling without stimulation. **(B)**. Cell distribution in three clustered phases of the cycle (G₀/G₁, S, and G₂/M) determined by DAPI staining. Cells in G₀/G₁ (*left peak*) showed about half of fluorescence intensity as cells in G₂/M state (*right peak*), and cells in S state (*middle peak*) presented a range of fluorescence between G₀/G₁ and G₂/M states. Representative results from two experiments are shown.

Figure 6.

Stimulated HSPC express mRNA for cytokines for augmenting and maintaining memory T cells in normal and lupus bone marrow. Purified LSK cells from seven BL/6 (A) and twelve lupus prone B/Wf1 mice (B) were cultured with R848 or CpG1585 for 16 hours followed by RNA extraction for qPCR in triplicates. Fold increases in relative expression is shown as compared to normal bone marrow B220⁺ cells which served as a background control. R848 and CpG1585 stimulated both normal and lupus LSK cells to express high levels of mRNA encoding cytokines and mediators which are related to Th17 induction and expansion, including TNF α , IL-1 β , IL-18, IL-6, IL-12 β , BAFF, and COX2. The stimulated LSK cells also expressed mRNA for IL-7 and IL-15 which are required for memory T cell maintenance, and high level of GM-CSF mRNA. These LSK cells were devoid of MKP (Results section 3.4).

Figure 7.

HSPC stimulated by apoptotic products and related TLR signals augment memory Th17 and Tc17 cells in normal bone marrow. (A) Gating strategy for CD4 and CD8 naïve and memory T cell subsets. It is notable, that cell fractionation and gating to analyze T cells is different from those used for Figures 1-6, as described in the text (**section 3.5**). In this example, bone marrow of normal C57BL/6 mice were fractionated for B220⁻ CD11b⁻ CD90⁻ CX3CR1⁻ CD41⁻ CD117⁺ LSKen cells, and then a mixture of the LSKen cells and CD90⁺ cell fraction that contained T cells, LT-HSC and some ST-HSC, were cultured without stimulant for 1½ days and then stained for FACS analysis. Single live cells were first gated as shown, followed by gating out of CD117⁺ cells. The CD117⁻ cells were then gated for CD3⁺ and CD4⁺ or CD8⁺. Naïve and memory T cell subsets are identified with the following

phenotypes: “Naïve” CD4 T cells are CD62L^{high}CD44^{low}, central memory CD4 T cells (CD4 T_{CM}) are CD62L⁺CD44^{high}, effector memory CD4 T cells (CD4 T_{EM}) are CD62L⁻CD44^{high}. “Naïve” CD8 T cells are CD62L^{high}CD44^{low}. The CD8 memory T cells are divided further into CD8 T_{CM} (CD62L⁺CD44^{high}), CD8 T_{EM} (CD62L⁻CD44^{high}), and CD8 T stem cell memory or CD8 T_{SCM} that are CD62L⁺CD44^{low/intermediate}. Expansion of memory T cells by stimulated HSPC are shown in **(B)** and **(C)**. LSK enriched (LSKen) or LSK cells from 12 normal BL/6 mice were co-cultured with bone marrow CD90⁺ cells in the presence of R848, CpG1585, nucleosomes and necrotic B cells. The frequencies of IL-17⁺ memory T cells were analyzed by flow cytometry. Data are shown as mean ± SEM. **(B)** CpG1585 increased IL-17⁺ “naïve” CD4 T and IL-17⁺ CD4 T_{CM} with LSKen co-cultures (co-cultures with LSK were not done in the case of CpG stimulation). R848 increased IL-17⁺ “naïve” CD4 T, IL-17⁺ CD4 T_{CM} and IL-17⁺ CD4 T_{EM} with either LSKen or with LSK co-cultures. **(C)** R848 increased IL-17⁺ CD8 T_{CM} and CD8 T_{SCM} with LSKen or LSK co-cultures, and IL-17⁺ CD8 T_{EM} increased significantly with LSKen co-culture. CpG stimulation increased IL-17⁺ CD8 T_{CM} and T_{SCM} with LSKen co-cultures and IL-17⁺ CD8 T_{EM} also increased significantly with the LSKen co-culture. Significant *p* values are shown, as in Figure 1B,C, and given in detail in Results section **3.5**.

Figure 8.

Megakaryocyte progenitor/bipotent megakaryocyte-erythroid progenitor cells (MKP/MEP, or MM cells) presented particulate apoptotic products to augment memory Th17 and Tc17 cells in lupus bone marrow. LSKen, MM and CD90⁺ cells were isolated from lupus prone mice and co-cultured with nucleosomes or necrotic B cells. The frequencies of IL-17⁺ memory T cells were analyzed by flow cytometry. Data are shown as mean and SEM. **(A)** In the presence of MM cells with antigen presenting ability, necrotic B cells significantly increased percentages of IL-17⁺ naïve CD4 T, CD4 T_{CM} and CD4 T_{EM}; and with nucleosomes, increases occurred in IL-17⁺ naïve CD4 T and CD4 T_{CM}. **(B)**. Necrotic B cells and nucleosomes also increased IL-17⁺ CD8 T_{CM} and T_{SCM}. **(C)**. Bone marrow MM cells from lupus-prone B/WF1 mice were further purified from bead fractionated CD117⁺ cells followed by CD41⁺ cell sorting as a CD41⁺CD117⁺ population. These bone marrow MM cells were more efficient than bone marrow CD11b⁺ cells or CX3CR1⁺ macrophage/DC progenitors; or splenic MM cells or splenic CD11c⁺ cells in processing and presenting nucleosomes to augment nucleosome specific autoimmune Th17 response in splenic T cells of B/WF1 lupus mice even at a 1:42 ratio of MM cells to T cells. Significant *p* values are shown as in Figure 1B,C, and given in detail in Results section 3.5.