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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). Surprisingly, normal HSPC themselves produced IL-17 and IL-21 after 1½ days of stimulation, and the best stimulators were TLR 7/8 agonist; HMGB1–DNA; TLR 9 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.

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 TLR 9 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 TLR 9 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

Abbreviations: 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/WF1, (NZB × NZW)F1; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{SCM}, stem cell memory T cells; HMGB1, High-mobility group box-1; ssDNA, single-strand DNA.

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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, and IFNα, 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-Il17a^{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 a 25-gauge needle, and then single cell suspensions were prepared after RBC lysis. Splenocyte single cell suspension was prepared by digestion with 1 mg/mL Collagenase IV (Worthington Biochemical, Lakewood, NJ) and 50 μg/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 the "Results" section (Section 3.1.1), and as explained there, commercially available LSK isolation kits, or flow cytometry-sorting was 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, PerCp-Cy5.5-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 50 ng/mL PMA, 500 ng/mL ionomycin and 1 × GolgiPlug-Brefeldin A solution at 37 °C for 4 h. 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. LSKen 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) 10 μg/mL, LPS 200 ng/mL, R848, 1 μM, and CpG oligonucleotides 1585, 5 μg/mL, for 36 h[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 h was optimal for the cytokine detection by ICS, as a 24 h culture was too early, and many cells died after stimulation in 48 h 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 5 μg/mL, HMGB1 100 ng/mL [32], or HMGB1–DNA complex (20 ng/mL ssDNA with 100 ng/mL HMGB1) [33,34]. Cells were stimulated with apoptotic cell products for 36 h, and IL-17 and IL-21 production was examined by flow cytometry. Apoptotic thymocytes were produced by incubating thymocytes with 0.5 μg/mL staurosporine (Sigma-Aldrich, St. Louis, MO) at a