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Human Megakaryocyte Progenitors Derived from Hematopoietic Stem Cells of Normal Individuals are MHC class II-Expressing Professional APC that Enhance Th17 and Th1/Th17 Responses

Ariel Finkelsztein^{a,1}, Alaina C. Schlinker^{b,1}, Li Zhang^{a,1}, William M. Miller^{b,c}, and Syamal K. Datta^{a,d,*}

^aDivision of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

^dDepartment of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

^bDepartment of Chemical and Biological Engineering, McCormick School of Engineering, Northwestern University, Evanston, IL 60208

^cRobert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL 60611

Abstract

Platelets, like stromal cells, present antigen only via MHC class I, but the immune potential of their progenitors has not been explored in humans. We derived CD34⁺CD117⁺CD41⁺CD151⁺ megakaryocyte progenitors (MKp) *in vitro* from mobilized peripheral blood hematopoietic stem and progenitor cells (HSPC) of normal subjects using culture conditions akin to bone marrow niche, or organs that support extramedullary hematopoiesis. The MKp expressed MHC Class II in contrast to platelets and functioned as professional APC *before they matured* further. Moreover, MKp constitutively expressed mRNA encoding mediators for human Th17 expansion, including IL-1, IL-18, IL-6, TGFβ, IL-23, BAFF, and COX2. MKp also expressed high levels of type I interferon and IRF5 mRNA. In contrast to platelets, MKp augmented the expansion of Th17, Th1, and potent Th17/Th1 double-positive cells in normal PBMC and CD4 line T cells from normal subjects or lupus patients. The Th cell augmentation involved pre-committed memory cells, and was significant although modest, because only non-cognate MKp-T cell interactions could be studied, under non-polarizing conditions. Importantly, the MKp-mediated expansion was observed in the presence or absence of direct MKp-T cell contact. Furthermore, MKp augmented Th17 responses against *Candida albicans*, a serious opportunistic pathogen. These results indicate an

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*Address Correspondence to: Syamal K. Datta, Division of Rheumatology, Northwestern University Feinberg School of Medicine, 240 East Huron Street, Chicago, IL 60611, USA Phone: (312) 503-0535 FAX: (312) 503-0994 skd257@northwestern.edu.

¹A.F., A.C.S. and L.Z. contributed equally to this work

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immunologic role of MKp in situations associated with extramedullary hematopoiesis and mobilization of HSPC.

Keywords

Antigen presenting cell; APC; Megakaryocyte progenitors; Hematopoiesis; Th17 cells; Th17/Th1 cells

1. Introduction

Development of the megakaryocyte (MK) lineage in hematopoietic differentiation is considered to be exclusively geared for production of platelets. However, megakaryocyte progenitors (MKp) may have other functions that remain to be defined. In the case of reactive extramedullary hematopoiesis that occurs in various infections, autoimmune inflammatory diseases or graft-versus host disease, megakaryopoiesis accompanies myelopoiesis in the vicinity of T and B cells in peripheral lymphoid organs [1]. Whether the extramedullary MKp play a role in the immune responses under these conditions or are there solely to produce platelets, is not clear. In this regard, we recently showed that cells resembling MKp and bipotent megakaryocyte-erythroid progenitors (MEP) are markedly expanded in spleens of lupus-prone mice and they can act as professional APC that efficiently present nuclear autoantigens to selectively induce a Th17 response, without requiring Th17-polarizing culture conditions [2]. An expanded population of similar MKp-like cells was also found in the peripheral blood of lupus patients, but their functional activity could not be characterized due to the rarity of such cells in the periphery. To assess the immune potential of this human population in the present study, we derived MKp *in vitro* from mobilized peripheral blood hematopoietic stem and progenitor cells (HSPC), using culture conditions akin to bone marrow niche or organs that support extramedullary hematopoiesis [3]. We found that the MKp were professional APCs that expressed MHC class II, in marked contrast to platelets, which express only MHC class I similar to stromal cells capable of presenting antigen only to CD8 T cells [4,5]. Indeed, surface MHC Class II expression diminished as the MKp matured. Moreover, in contrast to platelets, which suppress Th17 responses [6], we found that the MKp produced mediators that augmented Th17, Th1 and potent Th1/Th17 responses even in non-cognate interactions under non-polarizing conditions.

2. Materials and Methods

Unless otherwise specified, reagents were obtained from Sigma-Aldrich (St. Louis, MO), cytokines from Peprotech (Rocky Hill, NJ), and antibodies from BD Biosciences (San Jose, CA).

2.1. Hematopoietic stem cell culture for MKp production

Cryopreserved CD34⁺ HSPC were purchased from the Fred Hutchinson Cancer Research Center with Northwestern University Institutional Review Board approval. HSPC were obtained from nine healthy adult donors undergoing G-CSF mobilization following informed consent. HSPC were cultured for MKp differentiation and production, as described

previously [3]. Briefly, cultures of CD34⁺ cells were initiated at 50,000 cells/ml in tissue-culture-treated T-flasks with IMDM + 20% BIT (78% IMDM [Gibco, Carlsbad, CA, USA], 20% BIT 9500 Serum Substitute [STEMCELL, Vancouver, BC, Canada], 1% Glutamax [Gibco], 1 µg/ml low-density lipoproteins [Calbiochem]) supplemented with 100 ng/ml thrombopoietin (Tpo), 100 ng/ml stem cell factor (SCF), 2.5 ng/ml IL-3 (R&D Systems, Minneapolis, MN, USA), 10 ng/ml IL-6, and 10 ng/ml IL-11. Cells were cultured in a fully humidified chamber at 37 °C, 5% CO₂, and 5% O₂ (hypoxia conditions). CD41, CD34, CD151, and CD117 expression was assessed in the viable cell population (DAPI⁻; DAPI from Life Technologies, Carlsbad, CA) by flow cytometry (LSR II; BD Biosciences). On day 4, 5 or 6 of HSPC culture, MKp were enriched using anti-CD61-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany), then resuspended in fresh IMDM+20% BIT. CD61 is a beta 3 integrin that associates with CD41 (alphaIIb) to form the heterodimeric complex CD41/CD61 (gpIIb/IIIa) on the membrane of MK lineage cells. To confirm the presence of CD41⁺ MKp in the isolated cells, anti-hCD41a-eFluor450 (eBioscience, San Diego, CA) at 5 µl/test was used. The purified MKp were then used for co-culture with T cells. To assess expression of the MHC class II (MHCII) cell surface receptor HLA-DR on MK at different stages of maturation, CD61⁺ MK were stained with anti-HLA-DR-APC (eBioscience), anti-CD34-PE-Cy7, anti-CD41a-FITC, and anti-CD42b-PE.

2.2. Derivation and culture of short-Term CD4 T cell line

Short-term T cells lines were derived and cryopreserved as described [7,8]. Briefly, PBMC were isolated from fresh blood with Ficoll-PaqueTM Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), washed 3X with PBS, then resuspended at 1 x 10⁶ cells/ml in complete RPMI (cRPMI; RPMI 1640 with glutamine [VWR, Radnor, Pennsylvania], supplemented with 10% heat-inactivated fetal bovine serum [FBS], penicillin/streptomycin, 1 mM HEPES [Cell Gro, Manassas, VA], 4.5 g/l glucose and 50 µM β-mercaptoethanol) with 10 U/ml IL-2 (R&D Systems) for culture. To prepare wells coated with anti-CD3/anti-CD28, wells of a 24-well, flat-bottom plate were incubated with 500 µl/well of 10 µg/ml (1X) rabbit anti-mouse IgG (MP Biomedical, Santa Anna, CA) at 37°C for 1.5 hours or overnight at 4°C, washed with 1 ml of PBS, and then incubated with 500 µl/well of 1 µg/ml anti-CD3 and 0.5 µg/ml anti-CD28 for 1.5 hours or overnight at 4°C. Wells were washed with PBS prior to use. PBMC were then placed into the anti-CD3- and anti-CD28-coated wells, and cultured for 3–5 days with 20 U/ml of IL-2, 50 ng/ml of IL-7 and 50 ng/ml of IL-15 (all from R&D Systems), in a volume of 2 ml/well. To rest the cells after anti-CD3/anti-CD28 stimulation, the cells were harvested and the concentration adjusted to 1 x 10⁶/ml with fresh cRPMI supplemented with 20 U/ml of IL-2, 50 ng/ml of IL-7, and 50 ng/ml of IL-15, and cultured in uncoated wells. After 4–5 days of culture, CD4 T cells were isolated from the cultured PBMC using anti-CD4-conjugated microbeads (Miltenyi) according to the manufacturer's instructions, then resuspended at 1 x 10⁶ cells/ml in cRPMI supplemented with 20 U/ml IL-2 and 50 ng/ml IL-7. Cells were cultured for an additional 4–5 days, bringing the total culture time after anti-CD3/anti-CD28 to 8–10 days. Cells were cryopreserved and stored in liquid nitrogen. After thawing and reseeding the CD4 line T cells in culture, the medium was changed every 3 days by removing 1 ml of old medium without disturbing the cells at the bottom of the well, and then 1 ml of fresh medium with IL-2, IL-7, and IL-15 was added back.

2.3. MKp and T-cell co-culture

Cryopreserved CD4 line T cells or PBMC were thawed and rested in 6-well plates in cRPMI with 10 U/ml of IL-2 at 37°C and 20% O₂ (normoxia) for 3–4 days. In certain experiments, PBMC were enriched with anti-CD45RA microbeads (Miltenyi) before co-culturing. Except in the *Candida albicans* stimulation assays, all co-culture wells were coated as follows. 96-well plates were coated with 10 µg/ml rabbit anti-mouse IgG (MP Biomedical) for 1.5 hrs at 37°C, washed in PBS, then coated with 1 µg/ml of mouse anti-hCD3 and 0.5 µg/ml of mouse anti-hCD28 IgG solution (Beckton Dickinson) for 1.5 hrs at 37°C, followed by two PBS washes. At the start of co-culture, MKp were seeded at a concentration of 13,000 to 20,000 cells per well, and CD4 line T cells were added at a concentration between 35,000 to 50,000 cells per well in a final volume of 100 µl. For the transwell insert experiments or experiments where a receiver, 96-well plate (larger capacity) was required, a 300 µl final volume, 26,000 MKp, and 100,000 PBMCs or 70,000 CD4 line T cells were used. The ratio of CD4 line T cells to MKp was maintained constant in all experiments at 2.7:1. Next, 5 U/ml IL-2, 50 ng/ml IL-7, 50 ng/ml IL-15, 100 ng/ml Tpo, and 100 ng/ml SCF were added and the cells were incubated at 37°C at 5% O₂ for 5–6 days. On the day of harvest, cell stimulation cocktail (eBioscience) was added for final concentrations of 80 nM phorbol-12-myristate-13-acetate (PMA) and 1.34 µM ionomycin. Cells were treated for 4 hours. During the final 2 hours, protein transport inhibitor cocktail (eBioscience) was added for final concentrations of 10.6 µM brefeldin A and 2 µM monensin.

Co-cultures of the CD4 line T cells (40,000/well) were also done with a megakaryoblastic cell line CHRF-288-11 after differentiating and stimulating the CHRF cells (24,000 cells/well) with PMA and lipopolysaccharide (LPS; LPS-UP, InvivoGen, San Diego, CA), as described [9]. The co-cultured cells were harvested and processed as above.

2.4. Culture conditions for transwell insert experiments

CD4 line T cells (70,000) were added to the wells of a 96-well receiver plate (Millipore, Billerica, MA). After addition of 5 U/ml IL-2, 50 ng/ml IL-7, 50 ng/ml IL-15, 100 ng/ml Tpo, and 100 ng/ml SCF, an insert plate with filters (0.4 µm pore size) was placed on top of the media to prevent direct cell-cell contact. Freshly-selected, CD61⁺ MKp (26,000) were added to each insert. The total volume in each well was maintained at 300 µl. Cells were cultured for six days at 5% O₂.

2.5. Culture conditions for supernatant experiments

MKp were selected from HSPC cultures using anti-CD61-conjugated beads (Miltenyi) on day 5 and a fraction of these cells was analyzed by flow cytometry using anti-hCD34 and anti-hCD151 antibodies. MKp cells were reseeded in IMDM+20% BIT medium supplemented with 100 ng/ml Tpo and 100 ng/ml SCF and cultured at 5% O₂. On day 7, cells were harvested and pelleted and the supernatant was removed, passed through a 4-mm-diameter, 0.22-µm-pore polyvinylidene fluoride (PVDF) filter (GE Healthcare UK Limited, Buckinghamshire, UK) to remove any cell membrane debris, and frozen at –80°C. The pelleted cells were resuspended in fresh IMDM+20% BIT with Tpo and SCF and cultured at 20% O₂. Supernatant harvest, filtration, and cryopreservation were also performed on days 9 and 11 of culture. These supernatants were used separately to replace the media of CD4 line

T cells (40,000 cells) cultured in anti-CD3/anti-CD28 coated wells. The T cells were incubated at 5% O₂ for 6 days before analysis by flow cytometry with the indicated antibodies.

2.6. Flow cytometry for T cell analysis

Cultured CD4 line T cells or PBMC were incubated with either Fixable Viability Dye eFluor® 506 (eBioscience) or LIVE/DEAD® fixable green dead cell stain kit (Life Technologies). DAPI was used as a viability stain if no intracellular staining was to be done. Cell surface staining was performed by incubating cells with anti-hCD4-V450 (eBioscience) or anti-hCD4-PECy7 (Beckton Dickinson) for 30 min at 4°C in the dark. Cells were washed with PBS+ 0.1% sodium azide and either acquired or prepared for intracellular staining. Cells were fixed and permeabilized in fixation/permeabilization solution (Beckton Dickinson) and washed twice in permeabilization buffer before addition of anti-IL-17-APC (eBioscience) or anti-IFN γ -PE (eBioscience) for 30 min at 4°C in the dark. Cells were washed twice in permeabilization buffer and resuspended in cold PBS before acquisition. Samples were acquired using an LSR II flow cytometer. Analysis and compensation of the samples was done with FlowJo Software version 8.8.7 (TreeStar, Ashland, Oregon) using fluorescence-minus-one (FMO) samples to determine positive staining [10].

2.7. DQ Ovalbumin endocytosis

DQTMOvalbumin (DQ Ova; Molecular Probes, Eugene, OR) is ovalbumin conjugated to BODIPY FL (excitation/emission of the digested product: 505/515 nm), which is rendered fluorescent green following proteolytic processing in the endosome-lysosome of APC. MKp from 5- or 9-day-culture or THP1 cells (human monocyte line from ATCC, Manassas, VA) were seeded at 15,000 cells per well in a 96-well plate. DQ Ova was added to the wells at the concentrations indicated and incubated for two hours at either 4°C (negative control) or 37°C. Cells were washed with PBS+0.1% sodium azide four times to remove unbound DQ Ova, and DAPI was added 10 min before acquisition to allow for identification of dead cells. Samples were acquired using an LSR II flow cytometer.

2.8. Confocal microscopy analysis of MKp processing of DQ Ova

CD61⁺ cells in IMDM+20% BIT medium with 100 ng/ml Tpo and 100 ng/ml SCF were incubated with 90.9 μ g/mL DQ Ova at 37°C or 4°C for 2 hours. To stop the reaction and remove excess DQ Ova, cells were washed three times with cold PBS + 0.5% FBS + 0.1% sodium azide. Cells were incubated with mouse anti-CD41a for 30 minutes, then incubated with goat anti-mouse-IgG-Texas Red (Jackson ImmunoResearch, West Grove, PA) for 30 minutes. Stained cells were fixed with 3.7% paraformaldehyde (Polysciences, Warrington, PA) in PBS for 10 minutes, then stained with DAPI for 10 minutes. Cells were allowed to settle on poly-D-lysine-coated glass slides for 1 hour, then a glass coverslip was mounted using Fluoromount G (SouthernBiotech, Birmingham, AL). Slides were imaged using a 63X oil objective on an SP5 II laser scanning confocal microscope (Leica, Wetzlar, Germany).

2.9. *Candida* stimulation Assay

We purchased PBMC that had been “characterized” to be strongly positive for IFN γ response to *Candida albicans* (Cellular Technology Limited, Shaker Heights, OH). To stimulate “characterized” PBMC with clinical trial grade *Candida albicans* antigen (GreerLabs, Lenoir, NC), the PBMCs were thawed according to the manufacturer’s recommendation and washed in anti-aggregate medium (Cellular Tech Ltd). 100,000 viable PBMC were combined with 26,000 MKp in a total volume of 300 μ l in uncoated wells. *Candida albicans* antigen solution was prepared by resuspending the entire vial contents (400 μ g) in 400 μ l of IMDM+20% BIT medium and was used at a final concentration of 167 μ g/ml. *Candida albicans*-treated PBMC were incubated at 5% O $_2$ for 5 1/2 days in uncoated wells.

2.10. qRT-PCR measurement of mRNA for Th-inducing cytokines and inflammatory mediators

MKp were enriched on day 5 or 6 from HSPC cultures and incubated for one hour in IMDM +20% BIT with 100 ng/mL Tpo and 100 ng/mL SCF at 5% O $_2$. One million cells per well in a 6-well plate were either left untreated or treated with 10 ng/ml of LPS for 12 hours. Total RNA was isolated from live MKp, control Jurkat (human T cell lymphoma) cells, the CD41 $^-$ fraction of cells from the day 5–6 HSPC cultures, or PMA-stimulated THP1 (human monocyte $\rightarrow$ macrophage line) cells using TRIzol® (Invitrogen, Carlsbad, CA) chloroform method. Briefly, cells were washed in PBS, 500 to 1000 μ l of TRIzol® reagent was added to lyse the cells, then 200 μ l of chloroform per 1000 μ l of TRIzol® was added and the samples centrifuged at 13,000 rpm in a microcentrifuge with maximum brake. The aqueous phase was then transferred to a new tube, one volume of RNase-free 70% ethanol was added and extracted by passing this solution through Qiagen RNeasy Minicolumns (Qiagen, Venlo, Netherlands), according to the manufacturer’s recommendation. The integrity of the RNA was confirmed by visual observation followed by photographic observation of the 28s and 18s ribosomal bands on a 2% TBE-agarose gel. The first strand was prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using 500 ng of total RNA according to the manufacturer’s recommendations. PCR was performed with a 7300 Real Time PCR System (Applied Biosystems). Taqman® Gene Expression Master Mix (Applied Biosystems) was used with TaqMan® probes (Applied Biosystems). 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 [11].

2.11. Statistical analysis

Wilcoxon signed-rank and Student’s *t*-test were used. Results are expressed as mean $\pm$ standard error of the mean, and *p* values less than 0.05 were considered significant.

3. Results

3.1. Efficient generation of MKp from CD34 $^+$ HSPC of normal donors

In lupus-prone mouse strains, splenocytes with MKp phenotype that express CD41, CD151 and CD117, are potent APC for inducing pathogenic Th17 cells in response to nuclear autoantigens [2]. In order to assess the functionality of the human counterpart of this

recently reported murine population, CD34⁺ HSPC from nine normal adult donors were separately cultured for MKp differentiation in serum-free medium with SCF, Tpo, IL-3, IL-6, and IL-11 at 5% O₂ for 4–6 days. Consistent with previous observation [3], CD34⁺ HSPC expanded (Fig. 1A) and ~30% committed to the MK lineage to give rise to CD34⁺CD41⁺ MKp (Fig. 1B). By day 5, an average of 6 MK (CD41⁺) were generated per input CD34⁺ cell (Fig. 1C). The majority of HSPC-derived MK also expressed the primitive progenitor cell markers CD34 and CD117, as well as the tetraspanin CD151 (Fig. 1D), which has previously been found on cells of the MK lineage [12]. On day 4, 5, or 6, the culture was enriched for CD61⁺ cells to generate a pure MKp population (Fig. 1E), which was used for co-culture with T cells. CD61 is a beta 3 integrin that associates with CD41 (alphaIIb) to form the heterodimeric complex CD41/CD61 (gpIIb/IIIa) on the membrane of MK lineage cells. Thus, all CD41⁺ cells are captured by anti-CD61 beads without masking CD41. Importantly, a majority of MKs in the CD61⁺ selected fraction were positive for CD34 (Fig. 1E), indicating that they were MKp. With further culture, the selected CD61⁺ fraction retained CD41⁺ MK purity, as the cells matured to gain CD42b and lose CD34 expression. (Fig. 1F).

3.2. MKp express high levels of mRNA for type I Interferon and Th17-inducing cytokines/mediators

Among conventional APC, dendritic cells have been shown to express Th17-inducing cytokines [13–15]. In addition, MKp-like cells from mice with lupus express messages for Th17-inducing cytokines [2]. In order to test if MKp differentiated from HSPC of normal human donors could produce such Th17-inducing and other inflammatory cytokines, we isolated total RNA from day-5 or day-6 MKp to measure the transcripts. We used RNA from Jurkat, a human T cell lymphoma line that does not produce inflammatory cytokines as our base control, and for comparison, RNA from the CD41[−] population derived along with the MKp from the day 5–6 HSPC cultures that would contain myeloid/DC progenitors, as well as RNA from PMA-stimulated THP1 cells, a human macrophage cell line. MKp from three different donors tested (among the nine used in this study)-, expressed mRNA for Th17-inducing cytokines, such as IL-1, IL-18 IL-6, TGF- β , and IL-23; the first two of which being especially important for *human* Th17-induction [16,17] were expressed at high levels by the MKp, comparable to PMA-stimulated THP1 cells (Fig. 2A). Moreover, the MKp highly expressed mRNA for other mediators that have been implicated in Th17 induction (Fig. 2A, B), namely BAFF, and COX2 (PTGS2); the latter mediates PGE2 production [18,19]. Remarkably, the MKp also expressed high levels of messages for the main type I IFN genes, IFN α -1, IFN α -2, IFN α -4 [20], and also IRF5 (Fig. 2B, C and E); the latter has been implicated in many autoimmune diseases for augmenting IFN production, and for increasing autoantigen presentation and inflammatory cytokines for induction of Th1 and Th17 cells [21,22]. The increased expression of mRNA for the type I IFN genes in MKp was also evident when compared to PMA-stimulated human macrophage APC line, THP1, in addition to the negative control, Jurkat T cell lymphoma line (Fig. 2C). Furthermore, the differentiated MKp expressed high levels of the above messages even without LPS stimulation, which did not significantly change the expression levels (results not shown). We also compared cytokine mRNA expression levels in the MKp (CD41⁺) with the CD41[−] population putatively containing myeloid/DC progenitors, which were separated from the

MKp fraction in the day 5–6 HSPC culture harvest. MKp showed elevated expression of RNA encoding IL-1, IL-23 and TGF β ; in this case both populations had been stimulated by LPS for 12 hours (Fig. 2D).

3.3. MKp process antigen efficiently and express MHCII similarly to professional APC

Mkp-like cells from spleens of lupus-prone mice have been shown to process nuclear autoantigens, and also the model antigen ovalbumin fed as DQTM-Ovalbumin, which fluoresces green only upon proteolytic processing in the endosome/lysosome of APC, and thus can be used to assess antigen-processing ability. The human MKp derived from different donors, when incubated with DQ Ova showed a dose-dependent increase in fluorescence at 37°C, but not at 4°C (negative control), suggesting antigen-processing ability (Fig. 3A, C). The change in fluorescence intensity was highly significant but relatively less than that observed for THP1 cells incubated with DQ Ova under the same conditions (Fig. 3B). Confocal microscopy of DQ Ova fluorescence also demonstrated MKp antigen-processing ability. As shown in Fig. 3D, the presence of red signal (CD41) along with green (processed ovalbumin) supports the hypothesis that human MKp can process foreign antigens. Because MHC II expression is also a characteristic of professional APC, we examined MK surface expression of HLA-DR. The most immature MKp, which still express CD34 but do not express the mature MK marker CD42b, have high levels of HLA-DR (Fig. 3E, red histogram), but expression is lost as the MKp mature to gain CD42b expression (Fig. 3E, blue histogram) and lose CD34 expression (Fig. 3E, orange histogram). HLA-DR has been reported to be expressed on primitive burst-colony-forming-unit MKp, but not on mature MK, and platelets in stark contrast to MKp completely lack MHC class II [4,5,23]. These observations are in line with MKp-like cells found in the spleens of lupus-prone mice, which lose the capacity to present nuclear autoantigens to Th cells as they mature and lose CD117 (cKit) marker [2]. Human MKp also expressed message for lysozyme (Lyz) at high levels (Fig. 2B), similar to APC derived from the myeloid lineage. These results support the hypothesis that the human MKp may act as MHC Class II⁺ APC in cognate interaction with Th cells, although we did not have access to the T cells from the same donors from whom mobilized HSPC were obtained to generate the MKp used in this study.

3.4. MKp augment Th17 expansion in CD4 line T cells when co-cultured under hypoxic conditions

Since it was not feasible to test cognate interaction with autologous T cells, we tested first if the MKp derived from normal donors could augment Th17 response in a non-cognate manner when co-cultured with cryopreserved CD4 line T cells from normal or lupus patient donors, which had been previously derived for other studies under non-polarizing conditions [7], as specified in the Methods, section 2.2. We co-cultured CD4 line T cells without MKp, as controls, or with MKp for six days in anti-CD3/anti-CD28 coated plates under hypoxia (which resembles conditions *in vivo*), and with media supplemented by IL-2 (low-dose), IL-7, IL-15, SCF and Tpo.

Because of the low frequency of Th17 cells, concurrent anti-CD3/anti-CD28 stimulation is routinely used to study effects of other types of APCs, microbes or drugs in expanding pre-committed Th17 cells under non-polarizing conditions [13,24–27].

The results of cultures shown in Fig. 4A (left = control without MKp, right = with MKp) strongly suggest that normal T cells from short-term lines can be skewed towards Th17 expansion by MKp derived from a normal donor. We obtained similar results by co-culturing T cell lines from normal subjects and active lupus patients (n=9) with MKp from a different donor, with or without direct contact (shown later). The results from all the lines are shown in Fig. 5B; the lines designated by “L” in the first letter are from lupus patients.

Because these T cell lines were derived in the past under conditions that are unfavorable for Th17 polarization or expansion [7], some of them might have lost pre-committed Th17 cells, and thus did not respond to the MKp under non-polarizing culture conditions.

Examples of two strong responder T cell lines, 91307, and LB42 are shown in Fig. 4B. The fact that 91307 responded similarly to MKp from two different donors suggests that MKp derived from normal donors can produce cytokines and mediators that can be utilized by non-cognate CD4 line T cells to expand Th17 and Th1 cells, which is consistent with results using PBMC (below).

Control co-cultures of the CD4 line T cells with a megakaryoblastic cell line CHRF-288-11 that were stimulated with PMA and LPS as described [9], did not show the significant Th17 expansion observed with MKp co-cultures.

Furthermore, the MKp cells, which were cultured by themselves in all experiments and identified as surface CD41+ cells, did not show any intracellular cytokine staining for IL-17 or IFN γ .

3.5. Direct contact between CD4 line T cells and MKp is not necessary for augmenting expansion of Th17 and Th1 cells

Having established that MKp could promote expansion of Th17 cells, we tested whether direct contact would be necessary to induce Th17 under our experimental conditions. We used six active lupus and three normal CD4 T cell lines for co-culture with MKp from a different donor in trans-well cultures. The conditions varied slightly from the direct contact experimental conditions to accommodate larger volumes. Using the same media and cytokines in wells coated with anti-CD3 and anti-CD28, we co-cultured MKp with CD4 line T cells separated by an insert that prevented direct cellular interaction between the two cell types. Cells were cultured under hypoxia for six days, and with the same factors used above. Staining was done for surface CD4, and for intracellular IL-17 and IFN γ (Fig. 5A–C) or for IL-17 alone (Fig. 5D). The results shown in Fig. 5A,B indicate that direct interaction is not required to help augment expansion of Th17 (7 out of 9 lines) or Th1 cells (8 out of 9 lines), as shown by the increase in the percentage of either Th17 cells or IFN γ ⁺ Th1 cells. An example is shown for a normal line (60408), and for an active lupus patient's line (LB42) (Fig. 5A). Although, the augmenting effect of MKp was modest because of non-cognate interaction, statistical significance was established for the totals using a non-parametric test

(Wilcoxon signed-rank) (Fig. 5B) producing a $p < 0.001$ and $p < 0.02$ for Th1 and Th17, respectively. These results with the trans-well cultures strongly suggested that a direct interaction between MKp and CD4 line T cells was not necessary for helping Th17 or Th1 expansion. To add support to this hypothesis and to test if MKp could produce soluble factors at various points during maturation, we tested the effect of supernatants obtained from MKp culture derived from the same donor at days 7, 9 and 11 of differentiation on normal CD4 line T cells (line 91307) that had demonstrated positive Th17 and Th1 expansion with MKp from two different donors (shown in Fig. 4). The result shown in Fig. 5C indicates that Th1 cells were expanded by the MKp supernatant while Th17 cells were not, suggesting that MKp must be present during co-culture in order to efficiently help in expansion of Th17 cells. Perhaps MKp produce factors that have a short half-life or participate in continuous feedback with T cells that is important for Th17 expansion, even in a non-cognate interaction.

To further support the hypothesis that MKp skew CD4 line T cells through a mechanism that involves secretion of interleukins and other mediators, we incubated CD4 line T cells with a combination of anti-IL1 β and anti-IL23 subunit p19 (both from eBioscience), at 10 μ g/ml. The inhibition was partial (57% inhibition, data not shown), indicating that other cytokines and mediators are also involved in Th17 expansion.

3.6. MKp help expand Th17, Th1 and Th17/Th1 double positive cells in memory T cell population of normal PBMC

Having found that MKp could help in the expansion of Th17 and Th1 in CD4 line T cells, we hypothesized that this effect could also occur in whole PBMC. CD45RO⁺ memory T cells, unlike naïve CD45RA⁺ cells have been shown to be susceptible for expansion by inflammatory cytokines [24]. We therefore asked if MKp could expand pre-committed memory CD4 T cells from normal PBMC. Cryopreserved PBMC were thawed and rested for two days in media containing low dose IL-2, then enriched for CD45RO⁺ cells using beads conjugated to anti-hCD45RA to deplete the naïve T cells. Memory-T-cell-enriched PBMC (containing their own APC) were incubated with IL-2, IL-7, IL-15, SCF and Tpo for 6 days at 5% O₂, with or without MKp derived from a new donor. Statistical analysis showed that MKp could significantly induce the expansion of Th17, Th1, and Th17/Th1 double positive cells in normal PBMCs (Fig. 6A–B). All the samples showing an increase in Th17 cells in Fig. 6B contained Th17/Th1 double positive cells.

3.7. MKp potentiate Th17 response to pathogens in normal subject PBMCs

Th17 cells protect against *Candida albicans*, a serious pathogen causing disseminated infection in immunodeficient subjects [25,28]. To test if MKp obtained from a normal donor could synergize with *Candida* to further help in expansion of Th17 cells, even in non-cognate interactions, we cultured whole PBMC from different donors with *Candida albicans* purified antigen (clinical trial grade) for five and a half days, with or without MKp. Fig. 7 shows results from three PBMC samples indicating that MKp further promoted expansion of CD4⁺Th17⁺IFN γ -cells (Th17 single positive) (paired *t*-Test, $p < 0.05$) over that achieved by *Candida* alone. This result suggests that MKp can synergize in enhancing Th17 response against a common pathogen, even in a non-cognate interaction.

4. Discussion

We have established that human MKp derived from mobilized peripheral blood stem cells of normal individuals have the properties of professional APC and they can enhance Th17 or Th17/Th1 responses, including those to pathogens. Using a previously described method to mimic conditions *in vivo*, CD34⁺ HSPCs cultured in hypoxic conditions (5% O₂) in media supplemented with Tpo, SCF, IL-3, IL-6, and IL-11 expanded and committed to the MK lineage giving rise to CD34⁺CD117⁺CD41⁺CD151⁺ MKp with a stable phenotype. Approximately six MKp were generated per HSPC seeded in this culture system.

MKp from different donors expressed transcripts for Th17-inducing cytokines, including IL-6, TGF β and IL-23, but most pronounced was the expression of IL-1 and IL-18, which are especially important for *human* Th17-induction [16,17]. Moreover, MKp highly expressed mRNA for other mediators of Th17 induction, such as BAFF, and COX2 (PTGS2); the latter mediating PGE₂ production [18,19]. Interestingly, the MKp also expressed high levels of mRNA for the main type I IFN genes, IFN α -1, IFN α -2, IFN α -4 [20], and also IRF5. Hyperactivity of IRF5 has been associated with many autoimmune diseases in that it plays an important role in augmenting autoantigen presentation and inflammatory cytokine production for the induction of Th1 and Th17 cells [21,22]. These results suggest that plasmacytoid DC are not the only source of Type I IFN in diseases like lupus.

MKp are expanded in the periphery of lupus subjects, probably as a part of abnormal mobilization of HSC from BM, as well as extramedullary hematopoiesis [2,29]. In this study, MKp were differentiated from HSPC cultures with cytokines and growth factors in hypoxic conditions (5% O₂) resembling the bone marrow niche, but these conditions are also present in sites of extramedullary hematopoiesis associated with a variety of infections and inflammatory autoimmune diseases. Importantly, MKp constitutively expressed the above cytokine messages at high levels, and LPS stimulation did not increase the message levels further. The MKp were geared to help Th17 expansion without further stimulation likely because some of the cytokines and culture conditions used for HSPC-to-MKp differentiation matched conditions found during extramedullary hematopoiesis.

High-level expression of type I IFN and the cytokines also indicate a potential novel function of MKp in the bone marrow in addition to the production of platelets. Type I IFN produced by MKp would help in mobilizing HSC, and along with the other cytokines (IL-6, IL-1), in emergency myelopoiesis [30,31]. Moreover, mature megakaryocytes (MK) have been shown to produce APRIL and IL-6 helping in the survival of long lived plasma cells in the bone marrow, which are normally the source of protective antibodies, but are also responsible for persistent production of pathogenic autoantibodies in lupus even after anti-CD20-mediated B cell depletion therapy [32].

MKp took up and processed model antigen efficiently and similarly to professional APC, and expressed HLA-DR highly. The MHC class II expression was lost as the MKp matured to gain CD42b expression and especially, lose expression of CD34. HLA-DR has been reported to be expressed on primitive burst-colony-forming-unit MKp, but not on mature

MK or platelets [5,23]. Similarly, MKp-like cells found in the spleens of lupus-prone mice, lose the capacity to present nuclear autoantigens to Th cells as they mature and lose the CD117 (cKit) marker [2]. The human MKp in this study also expressed mRNA for lysozyme in high levels, which has been thought to be a signature marker for APC of myeloid lineage. Although *Lyz* knockout mice are widely used to study the role of macrophages in immune response (Jackson Labs, Bar Harbor, ME), our results suggest that changes observed in those studies could have also been caused by *Lyz* deficiency in MKp APC. Platelets can process and present antigen only via MHC class I to stimulate CD8 T cell responses [5]. Thus, platelets unlike MKp, are limited by their expression of only MHC class I, like stromal cells [4,5]. MKp express MHC class II and augment expansion of Th17 and potent Th1/Th17 cells in contrast to platelets, which actually suppress Th17 responses [6]. Therefore, in addition to influencing other hematopoietic progenitor cells in the bone marrow, the MK-lineage plays an important role in priming and enhancing adaptive immunity.

MKp could significantly help in the expansion of Th17, Th1, and Th17/Th1 double-positive cells in normal PBMC or CD4 T cell lines derived from normal subjects or lupus patients. The augmentation by MKp required no direct contact with the T cells and could be mediated in a non-cognate interaction by cytokines and mediators produced by the MKp. In addition, MKp induced the expansion of pre-committed memory T cells. Remarkably, the Th17/Th1 double-positive cells expanded by the MKp are found commonly in humans and are considered to be especially potent in both immunity and autoimmunity [26,33,34].

Using PBMC (containing their own APC) from *normal* donors, we found that MKp can synergize in boosting Th17 response against a serious pathogen, namely *Candida albicans*, even in a non-cognate interaction. Th17 cells protect against disseminated infection by *Candida albicans*, which is life threatening in immunodeficient subjects [25,28]. Furthermore, the Th17 augmentation by MKp would probably be more pronounced in cognate interaction with autologous T cells, especially from subjects with immunosuppression.

In conclusion, megakaryopoiesis that accompanies extramedullary hematopoiesis in response to infections and autoimmune inflammatory diseases is generally thought to be solely for the production of platelets. Our results with human cells show that MKp are a part of innate immune system playing a critical role in boosting adaptive immunity for protection against pathogens in normal subjects. MKp might also augment autoreactive Th cells, especially the highly pathogenic Th17/Th1 double positive cells that occur in subjects with lupus or immune thrombocytopenia [2,35]. The augmentation of Th17 cells under non-polarizing culture conditions was modest but significant, as it occurred in non-cognate interaction with T cells, since autologous lymphocytes were not available from the mobilized subjects from whom the MKp were derived. Nevertheless, the results indicate that MKp would likely also potentiate responses in neighboring T cells taking part in a defensive or pathogenic response in situations associated with extramedullary hematopoiesis.

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Abbreviations

MKp	megakaryocyte progenitor
MK	megakaryocyte
MEP	bipotent megakaryocyte/erythroid–progenitor
HSPC	hematopoietic stem and progenitor cells
HSC	hematopoietic stem cell
Tpo	thrombopoietin
SCF	stem cell factor
pDC	plasmacytoid dendritic cell
PMA	phorbol-12-myristate-13-acetate
LPS	lipopolysaccharide

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Biographies

Ariel Finkielstein PhD. has a Licentiate degree in Biology from the University of Buenos Aires, a Master of Science in Genetics and Molecular Biology from the University of Alberta (1999), and a PhD in Developmental Genetics from the University of Western Ontario (2009). He has done postdoctoral training at the University of Chicago (Pathology), the University of Illinois at Chicago (Multiple Sclerosis and Stem Cells), and Northwestern University (CD4T-Th17 and CD4T-IFN α regulation by non-cognate megakaryocyte precursor cells). His main interest has been cell signaling and clinical research.

Alaina Schlinker PhD. obtained BS in Chemical Engineering, University of Southern California, and PhD in Chemical and Biological Engineering, McCormick School of Engineering, Northwestern University (2014). Research work involves studies on adhesion molecules essential for initiation of proplatelet formation in megakaryocytes, and different ways to develop in vitro-derived platelet production from hematopoietic stem cells to generate a transfusable product.

Li Zhang, M.D. PhD. is Research Assistant Professor of Medicine-Rheumatology at Northwestern University School of Medicine, Chicago. She received MD from Suzhou University School of Medicine, Suzhou, China (1984), Master of Science in Immunology from Dong Nan University School of Medicine, Nanjing, China (1990), and Ph.D. in immunology from Saga University School of Medicine, Saga, Japan (2000). She did post-doctoral research at Northwestern University School of Medicine, Chicago. Her research interests include nucleosomal histone peptide epitopes for tolerance therapy of lupus and pathogenic circulating T_{FH} cells and APC in lupus patients and SLE mouse model.

William M. Miller PhD. is Professor of Chemical and Biological Engineering at Northwestern University. He obtained BS from Lehigh University, MS from MIT, and PhD from the University of California, Berkeley. His research is focused on expansion and controlled differentiation of hematopoietic stem and progenitor cells, culture surfaces that mimic aspects of the *in vivo* bone marrow niche, mechanisms that regulate hematopoietic cell differentiation, and bioreactor systems for hematopoietic cell and platelet production. He is a Fellow of AAAS and AIMBE, Editor of the *Biochemical Engineering Journal*, and served on the Scientific Advisory Boards of the Australian Stem Cell Centre and the Stem Cell Network of Canada.

Syamal K. Datta, M.D. is Professor of Medicine and Microbiology-Immunology at Northwestern University School of Medicine, Chicago. He has held Solovy Arthritis Research Society Professorship; received MERIT award from NIH; is member of Association of American Physicians, and Faculty of 1000. After graduating from Calcutta Medical College, India in 1967, he did post-doctoral research at Tufts University, Boston. In 1980's Datta group showed that pathogenic anti-DNA autoantibodies are encoded by genes of normal subjects. They also identified nucleosomal histone peptide epitopes for tolerance therapy of lupus, defined role of Cbl-b in hyperexpression of CD40L and COX-2 in lupus, and MKp as a new category of APC.

Highlights

- Human megakaryocyte progenitors (MKp) are MHC Class II expressing professional APC
- Human MKp express cytokines and mediators augmenting Th17 and Th1/Th17 response
- MKp augment Th17 response against *Candida albicans*, serious opportunistic pathogen
- Unlike MKp, mature MK and platelets express only Class I and do not augment Th17
- MKp probably play immunologic role in conditions with extramedullary hematopoiesis

FIGURE 1.

In vitro generation and selection of MKp. CD34⁺ HSPC cultured at 5% O₂ in serum-free medium supplemented with Tpo, SCF, IL-3, IL-6, and IL-11 replicate (A) and ~30% commit to the CD41⁺ MK lineage (B), resulting in the production of an average of 6 MK per input CD34⁺ cell by day 5 (C). The majority of HSPC-derived MK (CD41⁺) also express CD34, CD117, and CD151. In (D), CD41⁺CD151⁺ cells (black box) are plotted to show CD117 and CD34 expression (i), next to a quantitative plot of this data (ii). Selection with anti-CD61 magnetic beads results in a highly-pure MK population, in which a majority of the MK express CD34 (E). Further culture of the CD61-selected fraction retains MK purity, as the cells mature to gain CD42b and lose CD34 expression (F). Panels A, B, and C show the averages for 4 experiments with HSPCs from distinct donors. D and F show results from a representative experiment. E, shows the averages for 6 experiments with HSPCs from distinct donors.

FIGURE 2.

MKp constitutively express mRNA encoding Th17-inducing cytokines/mediators, Type I interferon, and IRF5. Fold increase in relative expression is shown as compared to Jurkat, a human T cell lymphoma line or PMA-stimulated THP1, a human monocyte/macrophage line (panels **A**, **B** and **C**). Cytokine mRNA expressed by MKp (CD41⁺) compared to CD41⁻progenitors derived along with the MKp from HSPC cultures is shown in panel **D**; both populations were stimulated by LPS only in this panel. Expression of RNA encoding Lyz and IRF5 by MKp are shown in **E**. Results from MKp derived from three different donors are shown. In this and subsequent figures donor number from whom the MKp were derived is designated by (#).

FIGURE 3.

MKp process antigen and express surface MHC II. MKp (**A**) or THP1 cells (**B**) were incubated with varying concentrations of DQ Ova for two hours at either 4°C or 37°C. Flow cytometry histograms for fluorescent DQ Ova are shown. MKp from another donor were incubated with DQ Ova for two hours at either 4°C or 37°C and analyzed by flow cytometry (**C**), or transferred to slides, fixed, and incubated with DAPI to stain nuclei before confocal microscopy (**D**). MK (CD41⁺) are positive for the MHC II cell surface receptor HLA-DR, and then gradually lose expression as they mature to gain CD42b and lose CD34 expression (**E**, dotted line = matched isotype).

FIGURE 4.

MKp in co-culture augment the expansion of Th17 and Th1 cells in CD4 T cell lines. **A.** CD4 line T cells were thawed and rested for three days with low-dose IL-2, then 50,000 live T cells were co-cultured with 20,000 live MKps in media supplemented with IL-2, IL-7, IL-15, SCF and TPO. After six days of incubation under hypoxic conditions, cells were stimulated with PMA-ionomycin and brefeldin/monensin, stained and analyzed for CD4, IL-17, and IFN γ expression by flow cytometry. Graphs in **A**, show the percentages of live, CD4 line T cells positive for IL17 and IFN γ in a control well and a well that received MKp. **B.** CD4 line T cells from a normal subject (91307) and a lupus patient (LB47) were cultured

with MKp derived from another donor, as above but, stained for only CD4 and IL-17. IL-17 and CD4 expression is shown for live, CD4⁺ cells.

FIGURE 5.

Direct contact between CD4 line T cells and MKp is not required for augmenting the expansion of Th-17 and IFN γ positive T cells. **(A)** CD4 line T cells were co-cultured with MKp in media supplemented with IL-2, IL-7, IL-15, SCF and Tpo for 6 days at 5% O₂. MKp and CD4 line T cells were separated by inserts with 0.4 μ m pores. After incubation, PMA/ionomycin was added for 4 hrs and monensin/brefeldin for 2 hrs before staining. Representative examples are shown for CD4 line T cells from a normal subject (left) and a lupus patient. **(B)**. Graphical representation of the flow cytometric data of all the lines tested as in **(A)** above. Statistical analysis of the data showed a significant difference of the

percentage of IFN γ ⁺ and IL17⁺ CD4 T cells when compared to controls. (C). CD4 line T cells were cultured in supernatants obtained from days 7, 9 and 11 of MKp cultures (MKp was from the same donor used in panel A. IL-17 and IFN γ expression in CD4 T cells are shown.

FIGURE 6.

MKp augment expansion of Th17, Th1 and Th17/Th1 double positive CD4 T cells in PBMC from normal subjects. **(A).** Cryopreserved PBMCs were thawed and rested for two days in media supplemented with low-dose IL-2, after which they were enriched for CD45RO memory cells and then co-cultured with MKp in media containing IL-2, IL-7, IL-15, SCF and Tpo at 5% O₂. Cells were stained with a live-dead fixable dye, followed by surface staining for CD4 and intracellular IL17 and IFN γ . **B.** Graphs show the percentage of total Th17 (top) or IFN γ (bottom) expressing CD4 T cells after six days of incubation for each control culture and MKp co-culture (P value using Wilcoxon signed-rank test).

FIGURE 7.

MKp augment Th17 response to *Candida albicans* in normal PBMC. **(A)** PBMC designated as "LP" were cultured for 5½ days at 5% O₂ alone with *Candida albicans* antigen or with MKp and the antigen. Media was supplemented with IL-2, IL-7, IL-15, SCF and Tpo. At the end of the culture period, cells were stimulated with PMA/ionomycin and brefeldin/monensin and stained with a live-dead fixable dye, followed by surface staining for CD4 and intracellular IL17 and IFN γ . **B.** Graph showing fold-increase in the percentage of CD4⁺Th17⁺IFN γ ⁺ (Th17 single positive) cells for the conditions shown in A, for PBMC from three subjects (Student-paired t -test, $p < 0.05$).