Megakaryocyte Progenitors Are the Main APCs Inducing Th17 Response to Lupus Autoantigens and Foreign Antigens

Hee-Kap Kang, Ming-Yi Chiang, Diane Ecklund, Li Zhang, Rosalind Ramsey-Goldman and Syamal K. Datta

*J Immunol* 2012; 188:5970-5980; Prepublished online 4 May 2012;
doi: 10.4049/jimmunol.1200452
http://www.jimmunol.org/content/188/12/5970

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/05/04/jimmunol.1200452.DC1

References
This article cites 70 articles, 34 of which you can access for free at:
http://www.jimmunol.org/content/188/12/5970.full#ref-list-1

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

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

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Megakaryocyte Progenitors Are the Main APCs Inducing Th17 Response to Lupus Autoantigens and Foreign Antigens

Hee-Kap Kang,* Ming-Yi Chiang,* Diane Ecklund,* Li Zhang,* Rosalind Ramsey-Goldman,* and Syamal D. Datta†,*‡

In search of autoantigen-presenting cells that prime the pathogenic autoantibody-inducing Th cells of lupus, we found that CD41+ CD151+ cells among Lineage– (Lin–) CD117+ (c-Kit+) CX3CR1– splenocytes depleted of known APCs were most proficient in presenting nuclear autoantigens from apoptotic cells to induce selectively an autoimmune Th17 response in different lupus-prone mouse strains. The new APCs have properties resembling megakaryocyte and/or bipotent megakaryocyte/erythroid progenitors of bone marrow, hence they are referred to as MM cells in this study. The MM cells produce requisite cytokines, but they require contact for optimal Th17 induction upon nucleosome feeding, and can induce Th17 only before undergoing differentiation to become c-Kit+ CD41+ cells. The MM cells expand up to 10-fold in peripheral blood of lupus patients and 49-fold in spleens of lupus mice preceding disease activity; they accelerate lupus in vivo and break tolerance in normal mice, inducing autoimmune Th17 cells. MM cells also cause Th17 skewing to foreign Ag in normal mice without Th17-polarizing culture conditions. Several molecules in MM cells are targets for blocking of autoimmunization. This study advances our understanding of lupus pathogenesis and Th17 differentiation biology by characterizing a novel category of APC. The Journal of Immunology, 2012, 188: 5970–5980.

S
ystemic lupus erythematosus (SLE, or lupus) is a major systemic autoimmune disease with complex genetically determined effects on the immune system. MHC class II genes are major determinants of lupus susceptibility, underscoring the importance of autoantigen presentation by class II molecules to autoreactive Th cells in initiating the disease (1). In lupus autoimmune, pathogenic IgG autoantibodies that fix complement and bind FcγR on inflammatory cells are produced with help from Th1 and Th17 cells that are specific for peptides from nucleosomes or ribonucleoproteins of apoptotic cells, and such Th cells also infiltrate vital organs (2–11). Macrophages (e.g., tingible body macrophage) and dendritic cells (DCs) are normally tolerant to apoptotic cell Ags (12), but they are activated to present such autoantigens after binding by FcγR to IgG immune complexes containing apoptotic cell-derived DNA/RNA, which then dually stimulate via their TLR and FcγR (13–18). Hence, to generate the activating IC, IgG-switched autoantibodies have to be made first by T cell help. Moreover, B cells become efficient APCs to Th cells preprimed by other APCs (19), and B cells can be stimulated by nuclear Ags synergistically via BCR and TLR after developing high-affinity somatically hypermutated receptors with T cell help (20, 21), otherwise anti-DNA B cells are inactivated (22, 23). Thus, conventional APCs are essential for disease progression, but it is unknown what initially primes autoimmune Th cells. We fractionated spleen cells of lupus-prone mice in search of such an APC.

Materials and Methods

Mice

NZB and SWR mice were purchased from The Jackson Laboratory (Bar Harbor, ME) to breed lupus-prone SNF1 [(SWR × NZB)/F1] hybrids (24). Female SNF1 mice, like BWF1, have high serum levels of IgG class anti-DNA and other anti-nuclear autoantibodies by 2 mo and spontaneously begin to develop severe lupus nephritis by 5 mo of age (25). The other lupus-prone recombinant congenic strain B6.Sle1,2,3 homozygous for the NZM2410 lupus susceptibility QTL (Sle1, Sle2, Sle3) on the normal C57BL/6J background (26), normal C57BL/6 (B6), and OTHI transgenic [B6.Cg-Tg(TcraTcrb)425Scb/J] with T cells specific for OVA peptide OVA123–139 on 1-A^b were from The Jackson Laboratory. Females were used, and all studies were approved by the institutional animal care and use committee.

Human subjects

Ten patients in clinical remission (all females; aged 27–63 y) fulfilling American College of Rheumatology revised criteria for SLE (27) and six normal (healthy) subjects (five females and one male; aged 22–46 y) were studied with approval by the institutional review board. Disease activity by Systemic Lupus Activity Measure (SLAM) (5, 28, 29) ranged between 1 and 8 or that by Systemic Lupus Disease Activity Index (SLEDAI) ranged between 0 and 2 (30) for the patients. Demographic data for the lupus patients including clinical and treatment status are shown in Table 1.

Cell isolation

CD90+, CD4+, CD8+, CD19+, γδ+ T, DX5+, CD117+ (c-Kit+), CD11c+, CD11c, Gr-1, and FceRI+ cells from spleens were purified by magnetic bead conjugated Abs (Miltenyi Biotec, Auburn, CA). Lin–c-Kit+ pure cells were isolated using anti-CD117 beads after depletion of the above markers.
twice. Mast cells were isolated by anti-FcεRI-FITC and anti-FITC beads. CD4+CD8− T cells were purified as before (10, 31). Cell subsets were >90% pure.

**ELISPOT assay**

ELISPOT plates were coated with capture Abs to IFN-γ or IL-17 (BD Biosciences, San Diego, CA) as published (8, 32). On the basis of optimal cell ratios established from dose-response curves, T cells (1 × 10^5) were cultured with APC subsets (0.25 × 10^5 each) from 5-mo-old SNF1 mice in the presence of nucleosomes or saline. Cytokine-expressing cells were detected at 24 h for IFN-γ or 48 h for IL-17. Lin−c-Kit+ T cells cultured by themselves without nucleosomes or with nucleosomes (10–30 μg/ml) produced 30 ± 5 and 34 ± 6 spots, respectively. T cells plus Lin−c-Kit− cells in coculture without nucleosomes produced 15 ± 8 spots (background), and so did T cells (1 × 10^5) cocultured with low numbers (0.05 × 10^5) of Lin−c-Kit+ cells even in the presence of nucleosomes. Th17 response in OTII transgenic naive T cells to OVA peptide OVA_{223-239} was measured after 72 or 120 h. All ELISPOT assays were performed without artificial polarization conditions or PMA-ionomycin. Nucleosomes were prepared as described (3, 4).

**Transwell experiments**

Because the Transwell culture system (96-well plate) required a larger volume of culture, we used twice the number of cells than for all other ELISPOT cultures. Lin−c-Kit+pure cells (0.5 × 10^5) were placed in Transwell chambers separated by a 0.4-μm permeable membrane (Corning Costar, Cambridge, MA) from the culture of T cells (2 × 10^5/well) from 5-mo-old SNF1 mice in anti-IL-17-coated ELISPOT plates. After 48 h of culture, IL-17-positive spots were analyzed. T cells and Lin−c-Kit+ cells without nucleosomes produced 23 ± 6 spots as background.

**Blocking of Th17 responses by anti-MHC class II Abs**

T cells (1 × 10^5) were cultured with APC subsets (0.25 × 10^5 each) from 5-mo-old SNF1 mice in the presence of anti-MHC class II Abs (clone M5/114; eBioscience, San Diego, CA) or isotype control upon stimulation with nucleosomes in IL-17 ELISPOT plates. Anti-MHC class II Abs were preincubated with APCs for 1 h before addition of nucleosomes. Blocking of Th17 response to nucleosomes by anti-MHC class II Abs was analyzed by comparing isotype control in the 48-h ELISPOT assay.

**Autoantibody quantitation**

IgG class autoantibodies to ssDNA, dsDNA, and histone and nucleosome (histone–DNA complex) were measured by ELISA (3, 4).

**Helper assay for IgG autoantibody production in vitro/ex vivo**

To detect autoantibody-inducing help, CD90^+ T (1 × 10^5/well) or purified CD4^+ T (0.25 × 10^5/well) cells from spleens were cocultured with B cells (1 × 10^5/well) and APCs (0.25 × 10^5/well) from 5-mo-old SNF1 mice in a 96-well plate for 7 d in the presence of 0.3, 1, 3, and 10 μg/ml nucleosomes. Culture supernatants were assayed for IgG Abs (8, 33).

For testing inhibition in vitro, T, B, and Lin−c-Kit+ MM cells (APCs resembling megakaryocyte and/or bipotent megakaryocyte/erythroid progenitors that are CD41+c-Kit+ and possess APC machinery) from spleens of 5-mo-old SNF1 were stimulated with nucleosomes (10 μg/ml) in the presence of anti-CD151 (1–100 μg/ml) or isotype control for 7 d and supernatants analyzed for IgG autoantibodies.

For testing inhibitory effect in vivo, 5-mo-old SNF1 mice were injected with anti-CD41 or isotype control (R&D Systems, Minneapolis, MN; details in the Results section). Splenocytes of injected mice were stimulated with nucleosomes (3 μg/ml) in culture for 7 d and then supernatant analyzed for IgG autoantibody production ex vivo.

**Adaptive transfer and lupus nephritis**

CD11b^− cells were first isolated from CD90/CD19^− splenocytes of 5-mo-old SNF1 mice; next, CD11c^+ cells were isolated from the remaining CD90/CD19^+CD11b^− splenocytes, and then CD117^+ cells were positively selected from Lin^− cells. Isolated cells were pulsed with 30 μg/ml nucleosomes for 1 h and then transferred (1 × 10^6 cells/mouse) i.v. into recipient mice three times at 2-wk intervals. There were six recipient mice per group except for the saline injection controls where 15 animals were monitored (since batches were being used concurrently for other experiments). The recipients were monitored weekly for proteinuria by using Albustix (VWR Scientific, Chicago, IL), as described (34), and sera collected a month later for IgG autoantibodies. Animals with persistent proteinuria greater than 100 mg/dl for 2 wk were sacrificed and nephritis graded as described (33, 34).

**Gene profiling microarray and analysis**

As detailed in this article, spleen cells from three batches of three 5-mo-old SNF1 female mice were used each time to obtain three independent isolations of each type of APC population. RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) from each APC after 6-h pulse with nucleosomes (20 μg/ml), and expression analysis in triplicate was performed at our Genomics Core Facility using Illumina Mouse WG-6 v2.0 Expression Beadchips, covering around 45,281 genes and expressed sequence tags, and then analyzed by our Bioinformatics Consulting Core. Before transformation and normalization, A/P call detection selected 20,890 out of 45,281 probes with p < 0.01 having valid signals (35). For each pair, Lin−c-Kit+CX3CR1^− versus Lin−c-Kit+cX3CR1^+, Lin−c-Kit+pure versus Lin−c-Kit+cX3CR1^+, Lin−c-Kit−CX3CR1^− versus DC, and Lin−c-Kit−pure versus DC, differentially expressed genes were identified using an ANOVA model with empirical Bayesian variance estimation (36), yielding 1619 genes on the basis of raw p value <0.01 and false discovery rate adjusted p value <0.05 and 1.5-fold change in expression level in at least one of the comparisons (Supplemental Fig. 3A); and out of these, 230 genes were significantly upregulated in all four pair comparisons (Supplemental Table I). Microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE36284 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36284).

**Real-time RT-PCR**

To measure mRNAs for Th17-inducing cytokines in APCs or Th17 cell-related molecules in responding T cells, Lin−c-Kit+pure or MM cells were incubated with nucleosomes or T cells were stimulated by Lin−c-Kit+pure or MM cells with nucleosomes (20 μg/ml), respectively, for various time points. mRNAs for Th17-inducing cytokines in APCs or Th17-related molecules in responding T cells were measured with primers as described (Applied Biosystems) (8).

**Phagocytosis**

Engulfment of noncarboxylated fluorescein-labeled latex beads (2.1 μm; Polyscience Warrington, PA) or CFSE (Invitrogen, Carlsbad, CA)–labeled and irradiated apoptotic thymocytes at various ratios, at 37°C for 6–72 h, by Lin−c-Kit+ or other APC was assessed by staining with allophycocyanin–anti-CD117 or PE–anti-CD11b before incubation with latex beads or apoptotic thymocytes for microscopy, but stained after the incubations for flow cytometry. Apoptotic thymocytes were prepared by gamma-ray irradiation (3000 rad) and stained with annexin V and propidium iodide after 6–12 h to confirm apoptosis.

**DQ-OVA endocytosis**

BODIFP-conjugated DQ-OVA (D-12053; Molecular Probes, Eugene, OR) exhibits bright-green fluorescence upon proteolytic processing. DQ-OVA (10 μg/ml) was added to Lin−c-Kit+ cells (5 × 10^5 cells) and incubated at 4°C or 37°C for 20 min to 2 h. Controls include 4°C with DQ-OVA or without DQ-OVA and 37°C without DQ-OVA, according to the manufacturer’s references. Cells were then washed and analyzed by flow cytometry.

**Flow cytometry**

Cells were stained with allophycocyanin-conjugated anti-CD117, FITC– or PE–anti-CD11b, FITC– or PE–anti-CD11c (BD Pharmingen), PE– or PE-Cy5–anti-CD41, PerCP/Cy5.5–anti-Gr-1 (eBioscience), PE–anti-CD151 (R&D Systems), PE– or PerCP/Cy5.5–anti-CD150, Pacific blue–DQ48 (BioLegend, San Diego, CA), or matched isotype controls at 4°C for 30 min, then acquired by LSR II with FACSDiva (Becton-Dickinson) at our Immunobiology Center core facility and analyzed using FlowJo (Tree Star, Ashland, OR). For sorting, c-Kit− cells were isolated from Lin− splenocytes by magnetic beads and then stained with FITC–anti–CD41 and PerCP/Cy5.5–anti–Gr-1 (eBioscience), PE–anti–CD151, allophycocyanin–anti–CD105, PE–Cy7–anti–Sca-1, and Pacific blue–anti–CD48 at 4°C for 30 min and sorted by MoFlo (Dako Cytomation, Carpinteria, CA) with Summit and FACS express 3 software (De Novo Software, Los Angeles, CA) at our Robert H. Lurie Comprehensive Cancer Center core facility. For identification of MM cells in human peripheral blood mononuclear cells (PBMCs), PBMCs were prepared by Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ) gradient of whole blood from lupus patients and buffy coats or whole blood from healthy donors. PBMCs were stained with Alexa Fluor 700–anti-CD3, eFluor–anti-CD19, ECD–anti-CD14, allophycocyanin–Cy7–anti-CD16, allophyc
ELISPOT assays (nucleosomes were measured at 24 and 48 h respectively in splenocytes with 35 mice. Lin–c-Kit+ cells cultured by themselves without nucleosomes or with nucleosomes (at 10–30 μg/ml) produced 30 ± 5 and 34 ± 6 spots, respectively, at background levels (representative examples shown later in Figs. 3A, 6C); and coculture of these APCs with T cells without nucleosomes produced only a few background spots (15 ± 8), as stated also in the figure legends and in Materials and Methods. Moreover, nucleosome-activated Lin–c-Kit+ pure cells did not express IL-17 message by quantitative PCR or microarray (Supplemental Table I).

Lin–c-Kit+ pure cells express requisite cytokines but are also contact dependent for optimal Th17 induction

Freshly isolated or SCF-cultured Lin–c-Kit+ pure cells from spleens of SNF1 mice expressed mRNAs of Th17-inducing cytokines (38–41) upon incubation with LPS or nucleosomes, which respectively increased IL-6 mRNA by 27- and 10-fold, TGF-β mRNA by 108- and 18-fold, TNF-α by 38-fold for both, IL-18 by 9- and 5-fold, and IL-1β mRNA by 126- and 90-fold at the 6-h time point, but the levels of IL-23/IL-12 (p40), TGF-β mRNA by 126- and 90-fold at the 6-h time point, but the levels of IL-23/IL-12 (p40), TGF-β, or TL1a (43) mRNAs did not increase (Fig. 2A, 2B, and data not shown). Therefore, Lin–c-Kit+ pure cells might also express other mediators facilitating expansion of precommitted Th17 cells in

**FIGURE 1.** Lin–c-Kit+ cells purified from lupus spleens (L*K+ pure) are the main inducers of Th17 response to nuclear autoantigens without undergoing further differentiation

We depleted spleen cells of lupus-prone SNF1 mice of cells with mature lineage markers, including conventional APC (CD90+, CD3ε+, CD19+, B220+, CD11c+, CD11b+, CX3CR1+, NK1.1+, Gr-1+, Ter119+, and FcεRI+ mast cells-depleted) and then isolated pure CD117+ (c-Kit+, or K+) cells from the lineage− (Lin−, or L−) cells (Supplemental Fig. 1A). These Lin–c-Kit+ pure cells, which had the morphology of hematopoietic progenitor cells, were almost pure cells express requisite cytokines but are also contact dependent for optimal Th17 induction
Lin^-c-Kit^+ pure cells express Th17-inducing cytokines and are equipped with APC machinery. (A and B) Lin^-c-Kit^+ pure cells expressed mRNA for Th17-inducing cytokines on LPS stimulation (A) or on pulsing with nucleosomes (B). Note: The levels of IL-6 and IL-18 mRNAs appear to be low, but this is to accommodate all in the scale: the actual increases at the 6-h time point were 27-fold (p = 0.000002) and 9-fold (p = 0.009) by LPS and 10-fold (p = 0.00023) and 5-fold (p = 0.0015) by nucleosome pulse, respectively. (C) Lin^-c-Kit^+ pure cells fed with nucleosomes were separated from cocultured T cells by Transwell membranes in IL-17 ELISpot plates, and the responses of the T cells are shown. (D and E) Lin^-c-Kit^+ pure cells express MHC class II and CD86 (D), and their ability to induce Th17 response to nucleosomes was blocked by anti-MHC class II Abs (E). (F) Lin^-c-Kit^+ pure cells efficiently process OVA (DQ-OVA). (G) Representative histograms of CFSE-labeled apoptotic cells engulfed (engulfment was confirmed by microscopy, see Materials and Methods; similar to Supplemental Fig. 2, also see Fig. 4B). ▼ indicates apoptotic cell engulfed by CD11b^+ (left panel) or Lin^-c-Kit^+ pure cells (right panel). (H) Percent of apoptotic cell engulfed by cell subsets in FACS analysis. Mean ± SEM of five separate experiments.

Addition to Th17 induction and priming (described later). Nucleosomes did not stimulate the Lin^-c-Kit^+ pure cells due to any LPS contaminant, as addition of polymyxin B or anti-TLR4 Ab did not inhibit Th17 response elicited by nucleosome-pulsed Lin^-c-Kit^+ pure APCs, and no differences in endotoxin levels were found between nucleosome preparation and PBS or culture media using an endotoxin detection kit (data not shown). Notably, Transwell cultures showed freshly isolated Lin^-c-Kit^+ pure cells are contact dependent for inducing nucleosome-specific Th17 response (Fig. 2C), indicating that these APCs induce Th17 by secreted and surface molecules working at close range.

Lin^-c-Kit^+ pure cells have APC machinery to efficiently process and present apoptotic cells and nuclear autoantigen particles

Like other APCs, Lin^-c-Kit^+ pure cells expressed MHC class II and costimulatory molecule CD86, and incubation with nucleosomes or TLR ligands LPS and CpG increased MHC class II on their surface. Anti-MHC class II Abs blocked Lin^-c-Kit^+ pure cell-induced Th17 responses significantly. The Lin^-c-Kit^+ pure cells phagocytosed latex beads and apoptotic cells, and they also could process foreign Ag OVA comparable to DCs, and microarray showed they express cathepsin G (Fig. 2D–H, Supplemental Fig. 2, Supplemental Table I).

Lin^-c-Kit^+ pure cells play a pathogenic role in development of lupus disease

Upon apoptotic cell or nucleosome feeding, Lin^-c-Kit^+ pure cells from female SNF1 mice induced Th17 cells belonging to different Th subsets that are known to induce pathogenic autoantibodies in lupus (10, 31), as detected by ELISpot assay and RORγt increase (44), and they also augmented help for autoimmunebody production along with expansion of T follicular helper (Thf) cells (Figs. 3, 4A–C). The CD4^-CD8^- Th17 cells induced by the Lin^-c-Kit^+ pure APCs (Fig. 3) could contain αβ and γδ TCR positive cells and NKT cells (10, 31, 45). To test the pathogenic role in vivo, Lin^-c-Kit^+ pure cells, CD11c^+ cells (CD11c^-CD11b^-low^+CD117^-low^ DCs), or CD11b^+ cells (containing CD11c^-low^+ DC subset and CD117^-low^ cells) were pulsed with nucleosomes and then adoptively transferred i.v. into 12-wk-old female prenephritic SNF1 mice three times at 2-wk intervals. Two weeks after the final transfer (18 wk of age), 50% of recipients of Lin^-c-Kit^+ pure cells and 30% of CD11b^+ cell-recipient mice developed severe nephritis, whereas DC recipients or saline controls did not (χ^2 test, p = 0.023; log rank test, p < 0.05). Recipients of DCs started to manifest nephritis only from 8 wk after the third transfer. The nucleosome-pulsed, in vitro-manipulated (activated) DC and CD11b^+ cell preparations probably caused nephritis by inducing autoimmune Th1 cells (as in Fig. 1) in vivo, consistent with other studies (46). Adoptive transfer of Lin^-c-Kit^+ pure cells resulted in marked increase of pathogenic IgG3 anti-nucleosome autoantibodies (p = 0.0038), and also IgG2a anti-dsDNA (p = 0.00036) and IgG2b anti-nucleosome autoantibodies in sera of recipient mice (Fig. 4D–F and data not shown). Thus, nucleosome-pulsed Lin^-c-Kit^+ pure cells are comparable to other professional APCs in accelerating lupus disease with only a few transfers. CX3CR1^-c-Kit^+ pure cells differentially express genes related to megakaryocyte progenitors

CD117 (c-Kit) is also expressed by macrophage/dendritic cell precursors (MDP), which also express the chemokine receptor CX3CR1 (47–49). Therefore, from T and B cell depleted spleen cells, we sorted out CD117^-CX3CR1^+, CD117^-CX3CR1^- (MDP), CD117^-CX3CR1^+ and CD117^-CX3CR1^- cell subsets, and then tested their abilities to induce Th1 and Th17 responses to nucleosomes. CD117^-CX3CR1^- cells were Lin^-c-Kit^+CX3CR1^- or L^-K^+CX^-, and they induced solely Th17 response, whereas CD117^-CX3CR1^+ cells induced only Th1 response, but CD117^-CX3CR1^+ (L^-K^C^-) cells induced high Th1 and Th17 responses (Fig. 5A–C). The L^-K^C^- cells of this study are very similar to L^-K^pure cells (Fig. 1), which are also CX3CR1^+, although isolated in a different way. Therefore, we compared gene expression profiles of nucleosome-pulsed, Th17-inducing APCs that were isolated in

FIGURE 2. Lin^-c-Kit^+ pure cells express Th17-inducing cytokines and are equipped with APC machinery. (A and B) Lin^-c-Kit^+ pure cells expressed mRNA for Th17-inducing cytokines on LPS stimulation (A) or on pulsing with nucleosomes (B). Note: The levels of IL-6 and IL-18 mRNAs appear to be low, but this is to accommodate all in the scale: the actual increases at the 6-h time point were 27-fold (p = 0.000002) and 9-fold (p = 0.009) by LPS and 10-fold (p = 0.00023) and 5-fold (p = 0.0015) by nucleosome pulse, respectively. (C) Lin^-c-Kit^+ pure cells fed with nucleosomes were separated from cocultured T cells by Transwell membranes in IL-17 ELISpot plates, and the responses of the T cells are shown. (D and E) Lin^-c-Kit^+ pure cells express MHC class II and CD86 (D), and their ability to induce Th17 response to nucleosomes was blocked by anti-MHC class II Abs (E). (F) Lin^-c-Kit^+ pure cells efficiently process OVA (DQ-OVA). (G) Representative histograms of CFSE-labeled apoptotic cells engulfed (engulfment was confirmed by microscopy, see Materials and Methods; similar to Supplemental Fig. 2, also see Fig. 4B). ▼ indicates apoptotic cell engulfed by CD11b^+ (left panel) or Lin^-c-Kit^+ pure cells (right panel). (H) Percent of apoptotic cell engulfed by cell subsets in FACS analysis. Mean ± SEM of five separate experiments.
those two ways, namely purified Lin–c-Kit\(^{+}\) (L–K\(^{+}\)) cells or the Lin–c-Kit\(^{+}\)CX3CR1\(^{−}\) (L–K\(^{+}\)C\(^{−}\)) cell subset, with Th1-inducing APCs, namely Lin–c-Kit\(^{+}\)CX3CR1\(^{+}\) (L–K\(^{+}\)C\(^{+}\)) subset, or CD11c\(^{+}\)CD117\(^{−}\)c-Kit\(^{−}\) (DCs). We purified CD11c\(^{+}\)CD117\(^{−}\) cells from total DCs to compare (and contrast) with the former two cell preparations because c-Kit\(^{+}\) (CD117\(^{+}\)) DCs that are present in total CD11c\(^{+}\) cells can induce nuclear autoantigen-specific Th17 cells, albeit to a much lower extent than the former (Fig. 1A, 1B, Supplemental Fig. 1B–E). Spleen cells from three batches of 5-mo-old SNF1 female mice per batch were used to perform three independent isolations of each cell subpopulation. By analyzing the functions of 230 genes that were significantly and differentially upregulated in all four pair comparisons (see Materials and Methods and microarray data in Supplemental Table I), the two Th17-inducing APC isolates, namely Lin–c-Kit\(^{+}\)CX3CR1\(^{−}\) (L–K\(^{+}\)C\(^{−}\)) cell subset or purified Lin–c-Kit\(^{+}\) (L–K\(^{+}\)) cells, highly expressed genes for transcription factors Gfi-1B, GATA1, GATA2, NF-E2, Meis1, and FIGE.

**FIGURE 3.** Lin–c-Kit\(^{+}\)pure cells induce Th17 response by different subsets of T cells and induce molecules expressed by Th17 cells. (A) Lin–c-Kit\(^{+}\)pure cells fed with nucleosomes (10 \(\mu\)g/ml) were cocultured as APCs with \(\gamma\delta\) T cells, CD4\(^{+}\) T cells, or CD4\(^{+}\)CD8\(^{−}\) T cell subsets fractionated from SNF1 splenocytes, and the responses of the T cells in IL-17 ELISPOT plates are shown, in addition to the IL-17 spots produced by Lin–c-Kit\(^{+}\) pure APCs cultured by themselves in presence of nucleosomes (APC alone). The cells were isolated from 5-mo-old female SNF1 mice. T cells and Lin–c-Kit\(^{+}\) cells cultured without nucleosomes produced 13 ± 6 spots as background. (B) Lin–c-Kit\(^{+}\) pure cells and T cells from SNF1 mice were cocultured in the presence of 20 \(\mu\)g/ml nucleosomes and then analyzed for relative expression of mRNA of ROR-\(\gamma\) (RORc), integrin \(\beta\)1 (Ig\(\beta\)1), integrin \(\beta\)4 (Ig\(\beta\)4), IL-23R, IL-22, and IL-21 by real-time PCR at various time points. T cells alone or Lin–c-Kit\(^{+}\) pure cells alone did not express these genes. Mean ± SEM of three experiments are shown, \(n = 15\) mice.

**FIGURE 4.** Lin–c-Kit\(^{+}\)pure cells increase production of IgG autoantibodies, expand Tfh cells, and play a pathogenic role in lupus disease. In vitro helper assay: (A) Lin–c-Kit\(^{+}\) cells (APCs) increased production of IgG autoantibodies by lupus T and B cells upon nucleosome stimulation in 7-d helper assay (see Materials and Methods), and (B) Lin–c-Kit\(^{+}\) cells also activated T cells and B cells to produce IgG autoantibodies in cocultures as shown after processing/presenting apoptotic thymocytes at different ratios. (C) Percent of Tfh cell expansion by Lin–c-Kit\(^{+}\) cells. Three days after coculturing Lin–c-Kit\(^{+}\) cells and T cells with nucleosomes or PBS, cells were stained for ICOS, CXCR5, and PD-1 to identify Tfh cells. Lin–c-Kit\(^{+}\) cells expanded Tfh cells in both CD4\(^{+}\) and \(\gamma\delta\) T cell populations upon stimulation with nucleosomes, but Tfh cells were not expanded without APCs (T only). Mean ± SEM of three experiments, \(n = 15\) mice. (D–F) Lin–c-Kit\(^{+}\)pure cells accelerate severe lupus nephritis on adoptive transfer and markedly increased pathogenic IgG autoantibody levels in sera of recipients (E, F). Arrows indicate the time of adoptive cell transfer. Number of mice/group = 6; except saline control group = 15. CD11b, CD11b\(^{−}\)CD11c\(^{low}\) or CD11c\(^{low}\) or \(\gamma\delta\); L–K\(^{+}\) pure, Lin–c-Kit\(^{+}\)pure cells; Nuc, nucleosomes.
with highly significant changes (3- to 22-fold, \( p < 0.000000000044 \)) and IL-17 responses \( (p = 0.000026) \) gated for CD117High+CD41+ cells in peripheral blood (not spleens), lupus patients (in clinical remission) also showed 4.8-fold increase in MM cells \( (p = 0.0000025) \) and 10.4-fold increase in CD151+high MM cells \( (p = 0.00026) \) gated for CD117HighCD41+ cells in peripheral blood compared with normal humans (Fig. 7E). There was no correlation between frequency of MM cells and the clinical activity scores (SLEDAI or SLAM) in these patients (Table I).

**MM cells break tolerance in normal mice and induce Th17 skewing in response to foreign Ags without polarizing culture conditions**

The MM cells from B6.Sle induced Th17-skewed responses to nucleosomes like SNF1, and remarkably they could induce Th17 differentiation in naïve, normal B6 (Fig. 7F) without the polarizing culture conditions required by other APC types (54). However, cells of the MM phenotype purified from large batches of B6 spleens lacked the ability to induce autoimmune Th17 (Fig. 7F). Remarkably, MM cells purified from B6.Sle mice could also elicit a Th17-dominant response to foreign Ag, OVA (OVA peptide323–339), in the T cells bearing receptors for OVA323–339 of transgenic mice of normal B6 background without artificial polarizing culture conditions or PMA–ionomycin stimulation. In contrast, splenic APCs of the OTII mice themselves failed to induce Th17 (Fig. 7G). Thus, MM cells are Th17-inducing APCs in general.

**Molecular targets in MM cells for blocking autoimmune response**

Lin–c-Kit+CX3CR1” cells expressed megakaryocyte/platelet related molecules (Fig. 5D, Supplemental Table I). By quantitative PCR and flow cytometry of differentially expressed gene products, we validated and narrowed down candidate molecules in MM cells, which could be tested to inhibit the c-Kit+ MM cells themselves from presenting autoantigens or producing known Th17-inducing cytokines and/or interfere with close-range (contact-dependent) interactions necessary for inducing Th17 cells, after nucleosome pulsing. We also tested whether blocking of the selected target molecule on MM cells pulsed with nucleosomes can suppress their ability to stimulate T cell helper function.

**FIGURE 5.** Lin–c-Kit+CX3CR1” cell subset expresses megakaryocyte progenitor genes. (A) Flow cytometry of c-Kit+ (CD117+) cells for CX3CR1 marker in CD90 CD19+ spleocytes of SNF1 mice. (B and C) The subsets of c-Kit+ cells (APCs) were isolated by sorting (designated by color key) and then analyzed for inducing IFN-γ (B) and IL-17 responses (C) in SNF1 T cells to nucleosomes. Mean ± SEM of five independent experiments. (D) Microarray analysis (average of triplicates): each row represents a gene, and each column represents each APC type. L’K’C’x”, Lin–c-Kit+CX3CR1” cells; L’K’Cx”, Lin–c-Kit+CX3CR1” cells. Genes with highly significant changes (3- to 22-fold, \( p \) as low as 0.000000000044) are shown.

Tal1 (SCL), all important regulators of megakaryocytogenesis found in megakaryocyte progenitor (MkP) or bipotent megakaryocyte-erythroid progenitor (MEP) cells (50, 51), as well as genes for other signature molecules expressed by megakaryocyte progenitors/platelet lineage, such as CD41, CD42, Pf4, CD151, and TPO receptor (mpl, CD110) (Fig. 5D, Supplemental Fig. 3A, Supplemental Table I).

**CD41+ c-Kit+ cells in Lin–c-Kit+ CX3CR1” APCs were responsible for inducing Th17 response to nucleosomes**

We further fractionated Lin–c-Kit+CX3CR1” cells by sorting them to CD41+ cells, hematopoietic stem cell (HSC), multipotent progenitor 1 (MPP1), multipotent progenitor 2 (MPP2), and CFU-erythroid progenitor (CFU-E) (Fig. 6A and Supplemental Fig. 3B), based on bone marrow (BM) progenitor subsets criteria (52, 53), and then analyzed their APC function in inducing Th17. Only the CD41+ cells induced very strong Th17 responses to nucleosomes (Fig. 6C), and because these CD41+Lin–c-Kit+CX3CR1” cells of lupus spleen contained MEP and MkP by CFU assay and could differentiate into megakaryocytes/platelets in the presence of TPO, we refer to them as MM cells (MkP and MEP-like cells that are CD41+c-Kit+ and possess APC machinery). Moreover, feeding the MM cells with nucleosomes resulted in increased production of platelet Pf4 (Fig. 6B–E, Supplemental Table I). Once the Lin–c-Kit+CX3CR1” subset’s CD41+ cells differentiated to become c-Kit+ CD41+, they could not present nuclear autoantigens to induce Th17 cells (these cells would be included in the c-Kit+CX3CR1” cell subset of APC in Fig. 5A–C and the cells in the right lower quadrant of Fig. 7A, described later).

Lin–c-Kit+CD151” CD41+ (MM) cells are expanded in different lupus-prone mice and lupus patients

Among other surface markers indicated by microarray, all CD41+ cells in the Lin–c-Kit+CX3CR1” subset (MM cells) highly expressed the tetraspanin CD151 (Figs. 5D, 7A, 7C, 7D, Supplemental Table I). In contrast, the c-Kit+CD41+ cells in the right lower quadrant of Fig. 7A, did not express CD151 in the mice or induce Th17 response to nucleosomes (11 ± 4 spots), and those cells would be included in all Lin–c-Kit+ APC preparations, none of which were able to induce a Th17 response (Figs. 1, 5, 7). The MM cells in lupus-prone SNF1 spleens were increased up to 49-fold more \( (p = 0.00000001) \) than in normal C57BL/6 (B6), increasing with age, but the SNF1 MM cells’ Th17-inducing ability was similar irrespective of age (Fig. 7B and data not shown). Similar to nucleosomes (8), whole apoptotic cells also stimulated Th17 response in splenocytes of lupus-prone SNF1 female mice \( (p = 0.0387 \text{ to } 0.000136) \), but not in normal SWR, which have very few MM cells (data not shown). Another lupus-prone strain, B6.Sle, also had up to 46-fold increase of MM cells \( (p = 0.0000021) \) compared with that of B6. Even though measurement was feasible only in peripheral blood (not spleens), lupus patients (in clinical remission) also showed 4.8-fold increase in MM cells \( (p = 0.0000025) \) and 10.4-fold increase in CD151+high MM cells \( (p = 0.00026) \) gated for CD117HighCD41+ cells in peripheral blood compared with normal humans (Fig. 7E). There was no correlation between frequency of MM cells and the clinical activity scores (SLEDAI or SLAM) in these patients (Table I).
FIGURE 6. c-Kit+/CD41+ cells are the Th17 inducers in the Lin−c-Kit+/CX3CR1+ cell subset. (A) Isolated Lin−c-Kit+/CX3CR1+ cells were further sorted into CD41+ cells, HSC (Sca-1+/CD150−/CD48−) and MPP1 (Sca-1+/CD150+/CD48+), MPP2 (Sca-1−/CD150−/CD48−), and CFU-E (Sca-1−/CD150−/FcyRIII/II CD105+low/−). (B) c-Kit+/CD41+ cells differentiated into megakaryocytes in 3-d culture with IL-3, TPO, EPO, and SCF (right bottom panel). Phase contrast of each subset on 3-d culture (top and middle panels) and Giemsa staining of MPP2 cells (left bottom panel; scale bar, 10 µm) and cultured CD41+ cells (right bottom panel; scale bar, 20 µm). Culture conditions for determining hematopoietic lineage potential are given in Materials and Methods. (C) Sorted c-Kit+/CD41+ cells [from (A)] exclusively induced Th17 response to nucleosomes when cocultured with SNF1 T cells. The sorted subsets of Lin−c-Kit+/CX3CR1+ cells are designated by color key, and Th17 responses are shown by bars. Mean ± SEM of five independent experiments. (D and E) Lin−c-Kit+/CD41+ cells differentiated into CD42+ cells and produce platelet-related molecule, Pf4. FACS shows Lin−c-Kit+/CD41+ cells differentiated into CD42+ cells in the presence of TPO but not in SCF alone (D). Nucleosomes feeding increased the production of Pf4 in Lin−c-Kit+/CD41+ cells (intracellular staining). PBS (black line) = 14.0% Pf4+ cells versus nucleosome (blue) = 55.4% Pf4+ cells in c-Kit+ (CD117+) cell gated population (E). Dotted line, isotype control.

Discussion

The series of experiments in this study show that among Lin−c-Kit+ cells depleted of conventional APCs and mast cells, the most efficient presentation of apoptotic cell nuclear Ags for inducing autoimmune Th17 cells is a property of the CD41+ CD117+ subset of c-Kit+/CX3CR1+ cells, which could be made to differentiate into megakaryocyte/platelet and erythrocytes with appropriate growth factors (similar to MkP and MEP in BM), but they lost autoimmune-presentation and Th17-inducing ability upon differentiation to c-Kit+/CD41+ cells. Thus we refer to this new type of APC resembling bone marrow MkP and MEP progenitor cells as MM cells because they are found in the lupus spleen with nuclear autoantigen-presenting function, express previously unreported marker molecules in addition to possessing Ag processing machinery, and are capable of producing requisite Th17-inducing cytokines and costimulators. The MM cells lack receptors for
FLT3 ligand and M-CSF (data not shown), besides lacking CX3CR1; thus they do not have myeloid or DC–macrophage progenitor markers (47–49). Moreover, the earliest progenitor-like cells in the lupus splenic Lin–c-Kit+ cells that resemble HSC and MPP1 and MPP2 cells of BM (52, 53) did not have Th17-inducing capability. Another variety of Lin–c-Kit+ APCs, totally different from MM cells in this study, are generated by deliberate stimulation with IL-25; they are multipotent progenitor-like Th2-inducing cells residing in GALT, and they differentiate into macrophage and granulocyte lineages (60). Th17-inducing DC subsets have been detected in other systems (61, 62), but c-Kit stimulation of the DC or PMA and ionomycin stimulation of the T cells was required to detect Th17 response in those cases in contrast to the Th17-inducing MM cells of this study. Moreover, the Th17-inducing ability of c-Kit+ DCs was miniscule compared with that of MM cells in this study under natural autoantigen-presenting conditions (Fig. 1).

Notably, MM cells express megakaryocyte progenitor molecules that are also expressed by platelets (Fig. 5D, Supplemental Table I). Platelets have been implicated in lupus pathogenesis by expressing CD40L, producing cytokines, and participating in immune complex-mediated inflammation (63, 64). However, the c-Kit+ MM cells from lupus subjects isolated by flow cytometry sorting or magnetic beads had the size and morphologic characteristic of nucleated mononuclear cells (Fig. 1, Supplemental Fig. 1), which would exclude or gate out platelets, and we found in this study that only the MM cells could uptake and present nuclear autoantigens and induce Th17 cells in a class II-dependent manner before undergoing further differentiation (Figs. 1, 2).

Table I. Lupus patient demographics: clinical and treatment status of SLE patients who provided samples for this study

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Age/Sex</th>
<th>SLEDAl</th>
<th>SLAM</th>
<th>Current Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54/F</td>
<td>0</td>
<td>Pred., HCQ</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38/F</td>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>47/F</td>
<td>2</td>
<td>AZA, LEF</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>51/F</td>
<td>4</td>
<td>HCQ, SSZ</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60/F</td>
<td>8</td>
<td>HCQ</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27/F</td>
<td>7</td>
<td>MMF</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>40/F</td>
<td>1</td>
<td>HCQ, Vit D</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>50/F</td>
<td>2</td>
<td>HCQ</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>63/F</td>
<td>3</td>
<td>HCQ</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34/F</td>
<td>2</td>
<td>HCQ</td>
<td></td>
</tr>
</tbody>
</table>

AZA, Azathioprine (Imuran); HCQ, hydroxychloroquine (Plaquenil); LEF, leflunomide (Arava); MMF, mycophenolate mofetil; Pred., prednisone or steroids; SSZ, sulfasalazine; Vit D, vitamin D.
issue, anti-CD41 injections that caused a significant decrease of nucleosome-stimulated IgG autoantibody production ex vivo by the splenocytes of the treated mice (Fig. 8D) would have also depleted platelets (58, 59), which could have helped autoimmune B cells by increased expression of CD40L. However, in lupus, both autoimmune Th and B cells hyperexpress CD40L because of intrinsic defects anyway (29, 65), and the cells from the treated animals in this study were tested for autoantibody production ex vivo in a 7-d assay.

Remarkably, Th17 induction and expansion by nucleosome-pulsed MM cells occurred without any Th17-polarizing culture conditions or PMA–ionomycin stimulation, which are generally used to detect Th17 cells in other systems. Moreover, contact was required for optimal autoimmune Th17 induction under these natural conditions indicating that MM cells, upon apoptotic cell or nucleosome feeding, require accessory molecules, such as CD151 working at close range for Th17 induction, in addition to producing known cytokines (IL-1, TGF-β, IL-6, TNF, as shown in Fig. 2A, 2B). The MM cells not only primed but also induced expansion of precommitted nucleosome-specific Th17 cells, which are increasingly seeded into the periphery of lupus mice probably due to inefficient deletion and autoreactive selection in the thymus (56, 66, 67).

Among the array of molecules expressed by MM cells (Supplemental Table I), we found that anti-CD151 blocked Th17 induction and autoantibody production (Fig. 8A–C). However, anti-CD151 did not inhibit the MM cells themselves (Fig. 8C). CD151 could facilitate interaction of MM cells with Th17 cells by increasing adhesion. Indeed, Th17 cells express the CD151 binding integrin α4β1 (56), whose expression was markedly augmented in the T cells when cocultured with MM cells pulsed with nucleosomes (Fig. 3B). Of course, CD151 or the other molecules on MM cells that could be targeted to inhibit Th17 induction, such as CD41, Pf4, and c-Kit (Fig. 8), are not uniquely specific for MM cells. Nevertheless, a combination of cytokines and surface molecules expressed by the MM cells make them potent Th17 inducers. Studies with the new type of Th17-inducing APC reveal new mechanisms for agents being beneficial in autoimmunity.

We have clearly shown that removal of Lin−c-Kit+CD41+ CD151+ cells completely abrogates Th17-inducing ability (Fig. 6C) and conversely that adoptive transfer of those cells fed with apoptotic nuclear Ags accelerate lupus disease (Fig. 4D). The preferential increase in IgG3 autoantibodies to nucleosomes by adoptive transfer of nucleosome-pulsed MM cells was striking, although pathogenic IgG2a and IgG2b autoantibody subclasses were also increased (Fig. 4E, 4F). IgG3 autoantibodies are especially pathogenic in lupus nephritis by forming cryoglobulin immune complex deposits (68, 69). Marginal zone B cells could also be involved in producing IgG3 anti-DNA autoantibodies in lupus.
that such patients are not in true immunological remission because are expanded in lupus patients in clinical "remission" confirms role in pathogenesis in the mouse models. The fact that MM cells NKT cells (31, 45), which are induced by the MM cells (Fig. 3A).


171: 3296–3302.


