Coactivator-Associated Arginine Methyltransferase 1 Regulates Fetal Hematopoiesis and Thymocyte Development

Jia Li, Ziqin Zhao, Carla Carter, Lauren I. R. Ehrlich, Mark T. Bedford and Ellen R. Richie

J Immunol 2013; 190:597-604; Prepublished online 17 December 2012; doi: 10.4049/jimmunol.1102513
http://www.jimmunol.org/content/190/2/597

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/12/17/jimmunol.1102513.DC1

References

This article cites 38 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/190/2/597.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
Coactivator-Associated Arginine Methyltransferase 1 Regulates Fetal Hematopoiesis and Thymocyte Development

Jia Li,*‡ Ziqin Zhao, ‡ Carla Carter,* Lauren I. R. Ehrlich,*1 Mark T. Bedford,*1 and Ellen R. Richie*†

Coactivator-associated arginine methyltransferase 1 (CARM1) is a protein arginine methyltransferase that methylates histones and transcriptional regulators. We previously reported that the absence of CARM1 partially blocks thymocyte differentiation at embryonic day 18.5 (E18.5). In this study, we find that reduced thymopoiesis in Carm1−/− mice is due to a defect in the fetal hematopoietic compartment rather than in the thymic stroma. To determine the cellular basis for impaired thymopoiesis, we examined the number and function of fetal liver (FL) and bone marrow cells. Despite markedly reduced cellularity of hematopoietic progenitors in E18.5 bone marrow, the number of long-term hematopoietic stem cells and downstream subsets was not reduced in Carm1−/− E14.5 or E18.5 FL. Nevertheless, competitive reconstitution assays revealed a deficit in the ability of Carm1−/− FL cells to contribute to hematopoiesis. Furthermore, impaired differentiation of Carm1−/− FL cells in a CARM1-sufficient host showed that CARM1 is required cell autonomously in hematopoietic cells. Coculture of Carm1−/− FL cells on OP9-DL1 monolayers showed that CARM1 is required for survival of hematopoietic progenitors under conditions that promote differentiation. Taken together, this report demonstrates that CARM1 is a key epigenetic regulator of hematopoiesis that affects multiple lineages at various stages of differentiation. The Journal of Immunology, 2013, 190: 597–604.

Hematopoietic stem cells (HSC) can self-renew throughout life and differentiate into all myeloid and lymphoid lineages (1). Epigenetic modifications are a driving force of this cellular differentiation. Because the genetic information of cells pre- and postdifferentiation is identical, epigenetic marks like DNA methylation and histone methylation are critical for facilitating the lock-in of a differentiated state (2). Epigenetic mechanisms function like a ratchet, allowing lineage-specific differentiation, but generally not dedifferentiation. There is an element of plasticity to cellular differentiation that can be forcibly reversed by small molecule epigenetic regulators and/or the overexpression of a few specific genes to generate an induced pluripotent stem cell state (3). Perhaps one of the best biological systems to study epigenetic changes that correlate with differentiation is hematopoietic cell development. Indeed, recently, genome-wide DNA methylation patterns were analyzed at each major stage of hematopoiesis, revealing clear epigenetic signatures for each cell lineage (4). Like DNA methylation, there are a number of reports that arginine methylation also plays a critical role in lymphocyte development and signal transduction (5).

Arginine methylation is a common posttranslational modification that subtly alters the function of its substrates (6). It does this in a number of ways: 1) Arginine methylation provides a docking site for Tudor domain-containing effector molecules (7–10); 2) it can also block protein–protein interactions, as in the case of certain SH3 domain-driven interactions (11); 3) arginine methylation can negatively regulate AKT-mediated phosphorylation, because the AKT consensus motif contains key arginine residues (12, 13); and similarly, 4) lysine methylation can also be blocked by adjacent arginine methylation events (14, 15). The substrates for protein arginine methyltransferases (PRMTs) are both nuclear and cytoplasmic, and in the nucleus, histones are a major target of these enzymes. Histone methylation allows the PRMTs to feed into the epigenetic code and contribute to key molecular switches that dictate cell fate.

The mammalian PRMT family of enzymes consists of nine members, PRMT1–9; the majority of these enzymes target the N-terminal tails for histones H3, H4, and H2A for methylation (16). Coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4 was the first family member to be identified as a transcriptional coactivator, which methylates the H3R17 and H3R26 sites, as well as other transcriptional regulators (6, 17). CARM1-null embryos display no overt developmental defects, although they are smaller than their wild-type counterparts, and once born, the nulls die without taking their first breath (18). In-depth analysis of these CARM1-null embryos has revealed a number of clear phenotypes, many of them associated with cell differentiation defects. CARM1-null lethality at birth is likely due to the fact that lungs from mice lacking CARM1 are inundated with immature alveolar type II cells, which do not develop into more mature alveolar type I cells; thus, CARM1 is required for the proper differentiation of alveolar cells (19). In addition, CARM1-null embryos lack brown
fat, and cells that do not express CARM1 are not able to differentiate into mature adipocytes (20). CARM1 is also required for chondrogenesis (21) and skeletal muscle development (22). Finally, we have also observed that functional CARM1 is required for normal T cell cellularity and differentiation (23, 24). Thus, there is genetic evidence that CARM1 activity impacts the differentiation of lung, fat, muscle, cartilage, and thymocytes.

We previously reported that CARM1 null embryos have a 5-fold reduction in thymocyte cellularity and a partial block in early T cell development (24). We now confirm a role for CARM1 in thymocyte development at the transition between the double-negative 1 (DN1) and DN2 stages. We investigated the cellular basis for impaired thymopoiesis in this mouse model. In this article, we report that CARM1 functions cell intrinsically to regulate hematopoietic progenitor cell activity and cellularity in the fetal liver (FL) and bone marrow (BM), respectively. Furthermore, when FL progenitors were cocultured on OP9-DL1 stroma, CARM1 was dispensable for T lineage differentiation in response to Notch ligands but was essential for survival. Taken together, these results demonstrate that CARM1 regulates fetal hematopoiesis and thymocyte development, bolstering the notion that epigenetic regulation is critical for proper differentiation and survival of multiple hematopoietic lineages.

Materials and Methods

Mice

Carm1−/− embryos were described previously (18) and generated by crossing Carm1+/− breeders. Timed pregnancies were established, and the day of vaginal plug was designated embryonic day 0.5 (E0.5). Embryos were genotyped using the primers 5′-CCACCTTTGTCATCTCCCT-3′ and 5′-TAATCTAAGGAAATGGATGG-3′. Mice transgenic for EFGP under control of the β-actin promoter were kindly provided by Dr. Irving Weissman (25). C57BL/6 and Rag2−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Thymic NCR+/mice were obtained from NCI (Frederick, MD). Mice were maintained in the Vivarium at the MD Anderson Cancer Center Science Park in accordance with guidelines established by the Association for the Accreditation of Laboratory Animal Care. All protocols using these mice were reviewed and accepted by the MD Anderson Animal Care and Use Committee.

Immunofluorescence microscopy

Serial sections (5 μm) from OCT-embedded frozen tissue were air dried and fixed in cold acetone for 5 min at room temperature. After washing in TNT (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.05% Tween 20), sections were blocked for 15 min in TNB (0.1 M Tris-HCL pH 7.5, 0.15% NaCl, 0.5% blocking reagent from TSA Biotin System [NEN Life Science Products, Boston, MA]). The slides were incubated at room temperature with primary Abs including rat anti-mouse CD45 (clone 30-F11; BD), polyclonal rabbit anti-cyto-keratin (Dako), rat anti-mouse CD25 (clone PC61; BD), rabbit anti-mouse K5 (Covance), and rat anti-mouse cKit (clone ACK45; BD) for 1 h. Secondary reagents included streptavidin-FITC (Vector Laboratories), Texas Red-conjugated donkey anti-rabbit IgG, and Texas Red-conjugated donkey anti-mouse IgG (Jackson Immunoresearch). In some cases, tyramide amplification was performed.

Flow cytometry

Single-cell suspensions were prepared from E18.5 thymi, spleen, and FL by dissociation of tissues through a 70-μm strainer (Fisher). Fetali BM cells were obtained by finely mincing E18.5 femur, tibia, humerus, radius, and ulna with a single-edged razor blade to release cells into FACS buffer. The dissociated cells and fragments were filtered through a 70-μm strainer (Fisher). BM and spleen cells were treated with RBC lysis buffer (17 mM Tris, 160 mM NH₄Cl, pH 7.3) to remove RBCs, then resuspended in FACS buffer (PBS pH 7.2, 0.005 M EDTA, 2% FBS). Cells were stained with fluorochrome-conjugated Abs in FACS buffer for 30 min on ice and washed with FACS buffer. Propidium iodide (Invitrogen) was added (0.5 μg/mL) to the sample immediately before data acquisition for cell exclusion. Anti-B220 (clone RA3-6B2) and anti-CD45 (clone 30-F11) conjugated to Pacific Blue, anti-CD27 (clone LG.3A10) conjugated to PerCP-Cy5.5, anti-CD4 (clone 129.20) conjugated to allophycocyanin, anti-e-c-kit (clone 2B8) conjugated to allophycocyanin-Cy7, and anti-FcyR (clone 93) conjugated to PE were purchased from BioLegend. Abs to CD90 (clone 53-6.7) conjugated to FITC or PE-Cy7, γδTCR (clone eBioGL3) conjugated to PE, CD3e (145-2C11), and CD127 (clone A7R34) biotinylated, CD4 (GK1.5) and TER-119 conjugated to allophycocyanin, c-Kit (clone 2B8) conjugated to allophycocyanin-eFluor 780, CD45 (clone 30-F11) conjugated to eFluor 450, CD25 (PC61.5) conjugated to Alexa Fluor 488, and CD34 (RAM34) conjugated to Alexa Fluor 700 were purchased from eBioscience. A lineage mixture containing Abs to CD3e (145-2C11) CD4 (GK1.5), CD90 (clone 53-6.7) CD11c (clone N418), CD11b (clone M1/70), B220 (clone RA3-6B2), TER-119, and Gr-1 conjugated to PE-Cy5 was purchased from Invitrogen. Biotinylated Abs were detected using streptavidin conjugated to Qdot 655 or allophycocyanin (Invitrogen). Cells were analyzed on an FACS Vantage B (BD Science), and data were analyzed using FlowJo software (Tree Star). The lineage mixture included Abs to B220, Ter119, Gr-1, Mac-1, NK1.1, and CD11c to exclude lineage-positive cells in thymocyte analyses. The lineage mixture included Abs to CD4, CD8, B220, Ter119, Gr-1, Mac-1, NK1.1 and CD11c to exclude lineage-positive cells in BM and FL analyses.

Kidney capsule transplantation

Fetal thymic lobes from E15.5 Carm1−/− or littermate controls were placed on Millicell 30-mm round 0.4-μm culture plate inserts (Millipore) such that they were floating at the liquid–air interface in RPMI 1640 medium (Invitrogen) containing 10% FBS (Atlanta Biologicals), 1% penicillin–streptomycin (Invitrogen), 2 mM l-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1.35 mM 2′-deoxyguanosine for 5 d. The thymocyte-depleted lobes were transplanted under the kidney capsule of athymic recipient NCrnu/nu mice.

Competitive repopulation assay

FL cells (2.5 × 10⁵) from Carm1−/− EGFp E14.5 embryos or controls (Carm1+/− EGFp or Carm1−/− littermates) were mixed 1:1 with competitor EGFp FL cells from wild-type C57Bl/6 E18.5 embryos and transplanted into sublethally irradiated (4.5 Gy) Rag−/− mice by retroorbital injection. The recipient mice were analyzed 8 or 16 wk after BM transplantation.

OP9-DL1 cocultures, proliferation, and apoptosis assays

Five hundred FACS-purified Lineage−/c-Kit−/Sca-1− (KLS) FL progenitors obtained from individual E14.5 Carm1−/− or control littermates were directly sorted into triplicate wells of 24-well plates containing a monolayer of OP9-DL1 cells in α-MEM + 10% FCS with 5 mg/mL Il-7 and 5 and nm/mL Fl3L (PeproTech). After 6 d in culture (37°C, 5% CO₂), hematopoietic cells were recovered and analyzed by flow cytometry for T cell differentiation markers. For DNA content analysis, live cells were stained with Vybrant Dye Cycle Violet (Life Sciences), according to manufacturer’s instructions. FlowJo software (Tree Star) was used to analyze the frequency of proliferating cells (S + G2/M), as determined by the Dean–Jett–Fox algorithm. Apoptosis was assessed by staining with Pacific Blue–conjugated Annexin V (BioLegend), according to manufacturer’s instructions, in conjunction with propidium iodide to detect dead cells. Forward and side scatter gates were set to tightly enclose live lymphocytes, thus excluding debris and small dead cells from our analyses.

Statistical analysis

P values from the Student t test on data distributed normally, whereas the two-tailed Mann–Whitney U test on data distributed normally. For comparison of the ratio of GFP+:GFP− to control or t test on data not distributed normally. The Kolmogorov–Smirnov test was first used to assess normality of the data in each experiment. To analyze the mean cell number in different hematopoietic subsets, we performed a Student t test on data distributed normally, whereas the two-tailed Mann–Whitney U test was performed on data that were not distributed normally. For comparison of the ratio of GFP+:GFP− mice, the one-tailed Mann–Whitney U test was used. A p value < 0.05 was considered statistically significant.

Results

Carm1 deficiency results in a block at the DN1-DN2 transition in thymopoiesis

We previously observed that CARM1 deficiency results in a reduction in thymic cellularity, associated with an accumulation of CD44+CD25−CD4−CD8− thymocytes at E18.5 (24). This pop-
ulation is heterogeneous, containing both T and non-T lineage progenitors. c-Kit is expressed on the most immature T cell progenitors, DN1 cells, within this population (DN1: c-Kit^+ CD44^+ CD25^+ CD4^+ CD8^+). DN1 thymocytes give rise to DN2 progenitors (c-Kit^-CD44^-CD25^-CD4^-CD8^-) that subsequently downregulate CD25 to become DN3 cells (c-Kit^-CD44^-CD25^-CD4^-CD8^-) that are committed to the T cell lineage. To further characterize the block in thymocyte development observed in Carm1^−/− embryos (24), we analyzed c-Kit expression within the CD44^-CD25^-CD4^-CD8^- compartment to distinguish the earliest T cell progenitor population. Consistent with our previous report, there was an increased frequency of CD44^-CD25^- thymocytes in the CD4^-CD8^- compartment in Carm1^−/− E18.5 embryos (Fig. 1A). Furthermore, analysis of c-Kit expression revealed an increase in the frequency of DN1 versus DN2 progenitors in Carm1^−/− E18.5 embryos, consistent with a block in differentiation of the earliest thymocyte subset.

The absolute number of DN1 thymocytes was not significantly different in Carm1^−/− E18.5 versus control embryos. However, the DN2 subset was significantly reduced, consistent with a block in the DN1-DN2 transition (Fig. 1B). Carm1 deficiency resulted in a ∼90% reduction in the number of DN2, DN3, and DN4 (c-Kit^-CD4^-CD8^-CD44^-CD25^-) thymocytes. DN4 cells are the immediate precursors of double-positive (DP) thymocytes (CD4^-CD8^-), which then give rise to mature CD4 single-positive (SP; CD4^-CD8^+) and CD8SP (CD4^-CD8^-) subsets. These latter subsets were reduced by ∼75% in the absence of Carm1 (Fig. 1B). We also observed a significant reduction of 85% in γδ T cells, as might be expected from a reduction in their progenitors, DN2 cells (Fig. 1B). Together, these data indicate that CARM1 is required for continued maturation of thymocytes beyond the DN1 stage.

**Reduced thymopoiesis in Carm1^−/− mice is not due to thymic stromal defect**

Thymopoiesis requires input from stromal cells in the thymic microenvironment. Thus, the block in thymocyte development observed in Carm1^−/− embryos could be an indirect consequence of a defect in the thymic stromal compartment. To determine whether Carm1 is required for proper thymic stromal function, we transplanted E15.5 2-deoxyguanosine–treated Carm1^−/− versus control fetal thymic lobes under the kidney capsule of athymic nude recipients. Eight to 12 wk after transplantation, thymic grafts were recovered and analyzed by flow cytometry. In contrast with the developmental block observed in Carm1^−/− E18.5 thymi (Fig. 1), thymopoiesis was not impaired when Carm1 deficiency was restricted to thymic stromal cells (Fig. 2). The cellularity was comparable for all thymocyte subsets regardless of whether they developed in a control or Carm1^−/− stromal environment. Engrafted Carm1-deficient lobes were smaller than controls at the outset of the transplantation experiment; this initial difference in thymic size could account for the slight decreases in thymocyte numbers observed in transplanted Carm1^−/− lobes (Fig. 2). In addition, comparable numbers of CD4SP and CD8SP T cells were recovered from the spleens of athymic recipients transplanted with Carm1^−/− or control fetal thymus (Supplemental Fig. 1). Altogether, the normal differentiation of thymocyte progenitors in a Carm1-deficient microenvironment, in contrast with the severely reduced cellularity in Carm1^−/− E18.5 thymi, indicates that loss of Carm1 predominantly affects thymocyte progenitors as opposed to the thymic stromal microenvironment.

**CARM1 deficiency affects thymocyte cellularity in the E12.5 thymic rudiment**

Given the defect in thymic cellularity at E18.5, we examined cryosections from E12.5–E18.5 from Carm1^−/− embryos to determine whether the reduction in thymocyte cellularity was apparent earlier in ontogeny. At E12.5, there was a striking paucity in the number of CD45^-Lin^- thymocytes from E18.5 Carm1^−/− and Carm1^+/+ or Carm1^-/- littermate controls. Arrows indicate gating strategy. DN thymocyte subsets are specified. Dot plots were depicted at low resolution when subset cellularity was low. (B) Cellularity of thymocyte subsets from E18.5 Carm1^−/− embryos and littermate controls. Each point represents an individual embryo. Mean values are indicated by the horizontal bars. Results are combined from four independent experiments with two or more mice per experiment. **p < 0.01, ***p < 0.001.

**FIGURE 1.** CARM1 deficiency results in an early thymopoiesis block in E18.5 embryos. (A) Representative flow cytometric analysis of CD45^-Lin^- thymocytes from E18.5 Carm1^−/− and Carm1^+/+ or Carm1^-/- littermate controls. Arrows indicate gating strategy. DN thymocyte subsets are specified. Dot plots were depicted at low resolution when subset cellularity was low. (B) Cellularity of thymocyte subsets from E18.5 Carm1^−/− embryos and littermate controls. Each point represents an individual embryo. Mean values are indicated by the horizontal bars. Results are combined from four independent experiments with two or more mice per experiment. **p < 0.01, ***p < 0.001.

**FIGURE 2.** CARM1 deficiency in the thymic stromal compartment does not impair thymocyte development. E15.5 Carm1^−/− or littermate control thymic lobes were incubated in 2-deoxyguanosine for 5 d and transplanted under the kidney capsule of athymic recipients. After 8 wk, thymocytes recovered from the grafts were analyzed by flow cytometry. Data shown are from five individual experiments with two or more animals per experiment. Mean values are indicated by the horizontal bars. *p < 0.05, **p < 0.01, ***p < 0.001.
DN1-DN3 thymocytes (Fig. 3B, 3C). By E13.5, Carm1<sup>+/−</sup> thymic lobes were markedly smaller than wild-type and remain hypoplastic, containing fewer thymocyte progenitors throughout embryogenesis (Fig. 3B, 3C). Thus, CARM1 plays a significant role in thymocyte development before and after thymic vascularization, which occurs at ~E14.5.

Early hematopoietic defect in Carm1<sup>+/−</sup> BM

Given the profound reduction in DN subsets at E18.5 (Fig. 1), together with the decrease in thymocyte progenitors at early stages of thymic organogenesis (Fig. 3), we hypothesized that the absence of Carm1 might affect prethymic hematopoietic progenitors. Because hematopoietic progenitors are present in the fetal BM by E18.5 (28), we compared E18.5 BM from Carm1<sup>+/−</sup> and control littermates for hematopoietic progenitor subset composition. Cellularity was reduced in Carm1<sup>+/−</sup> BM compared with littermate controls (data not shown). Interestingly, the proportion and number of c-Kit<sup>+</sup> cells within the lineage<sup>−</sup> fraction was greatly reduced in CARM1-deficient embryos (Fig. 4A, left panels, 4B).

The KLS progenitors, which contain the most undifferentiated hematopoietic progenitors, are reduced in both frequency and absolute number in the E18.5 Carm1<sup>+/−</sup> BM (Fig. 4). The KLS population consists of several progenitor subsets: long-term HSCs (LT-HSCs: Slamf1<sup>+</sup> Flk2<sup>−</sup> KLS), short-term HSCs (ST-HSCs: Slamf1<sup>−</sup> Flk2<sup>−</sup> KLS), and lymphoid-biased multipotent progenitors (Flk2<sup>+</sup>MPP: Flk2<sup>+</sup>Slamf1<sup>−</sup> KLS). All three of these early hematopoietic progenitors are significantly reduced in the E18.5 Carm1<sup>+/−</sup> BM. Flk2<sup>+</sup>MPP are reduced by 95%, whereas LT-HSCs and ST-HSCs were reduced by ~80% (Fig. 4).

In addition to the decrease in oligopotent hematopoietic progenitors in E18.5 Carm1<sup>+/−</sup> BM, downstream lineage-restricted progenitors were also diminished. The lineage<sup>−</sup>CD27<sup>+</sup>Flk2<sup>+</sup> BM subset contains all progenitors with thymocyte differentiation.

**FIGURE 3.** Carm1<sup>+/−</sup> fetal thymi contain fewer thymocyte progenitors than Carm1<sup>+/+</sup> littermates. Transverse cryosections of Carm1<sup>+/−</sup> and Carm1<sup>+/+</sup> fetal thymi were costained for thymocyte and thymic epithelial cell (TEC) markers as indicated. (A) Distribution of CD45<sup>+</sup> progenitors in E12.5 thymus rudiment. Scale bar, 100 μm. (B) Distribution of c-kit<sup>+</sup> (DN1 or DN2) thymocytes in E13.5 thymus rudiment (denoted by dashed line). Scale bar, 200 μm. (C) Distribution of CD25<sup>+</sup> (DN1 or DN2) thymocytes and K5<sup>+</sup> TECs in E13.5 (scale bar, 100 μm), E15.5 (scale bar, 500 μm), and E17.5 thymi (scale bar, 1000 μm). Three experiments were performed with thymi from two Carm1<sup>+/−</sup> and one Carm1<sup>+/+</sup> embryo per experiment. Sections were mounted in VECTASHIELD mounting medium (Vector Laboratories), and staining was analyzed at room temperature using an Olympus ProVis AX70 microscope with UPlanFl 40/0.75, UPlanFl 20/0.50, and UPlanFl 10/0.30 objectives, DP Controller and DP Manager software.

**FIGURE 4.** CARM1 deficiency results in reduced cellularity of hematopoietic progenitors in E18.5 BM. (A) Representative flow cytometric analysis of Lin<sup>−</sup> BM cells from E18.5 Carm1<sup>+/−</sup> null mice and littermate controls. Top row is a representative stain from C57B16J adult BM, which was used to define gating strategy for progenitor populations. Arrows indicate gating strategy. Hematopoietic subsets are specified. Dot plots were depicted at low resolution when subset cellularity was low. (B) Cellularity of BM progenitors from E18.5 Carm1<sup>+/−</sup> embryos and littermate controls. Each point represents an individual embryo. Mean values are indicated by the horizontal bars. Results are combined from five independent experiments with two or more animals per experiment. *p < 0.05, **p < 0.01, ***p < 0.001.
potential (29). Interestingly, this population was severely reduced by 93% in Carm1−/− BM. The CD27+Flik2+ compartment contains the lymphoid-restricted common lymphoid progenitor subset (CLP: Lineage−/CD27+Flik2+IL-7R−), which was reduced by 85% (Fig. 4). We also observed a reduction in myeloid-restricted progenitors (MP, cKit+ Lineage−/Sca1−) in the absence of CARM1 (Fig. 4). These progenitors can be subdivided into common myeloid progenitors (Lineage−/CD34+/FcgR+MP), further downstream granulocyte-macrophage progenitors (GMP: Lin−/CD34+/ FcgR+MP), and megakaryocyte/erythroid progenitors (MEP: Lin−/ CD34−FcgR−MP). Common myeloid progenitors and MEPs were significantly reduced by 92 and 66%, respectively, whereas neither BM GMPs nor splenic granulocytes were significantly diminished (Fig. 4, Supplemental Fig. 2). The reduction in LT-HSCs and subsequent progenitors through CLPs, together with our observations that E18.5 thymocytes and splenic B cells are reduced in Carm1−/− embryos (Fig. 1, Supplemental Fig. 2), suggest that CARM1 is required for early stages of hematopoiesis and continued lymphoid differentiation.

Cellularity of hematopoietic progenitors is not decreased in Carm1−/− FL

Although hematopoiesis is shifting to the BM by E18.5, hematopoietic progenitors are still present in the FL (28). Therefore, we analyzed the hematopoietic progenitor compartments from E18.5 FL in CARM1-deficient mice and littermate controls. In contrast with E18.5 BM, the number of FL cells in Carm1−/− mice is comparable with controls (data not shown). Interestingly, the number of LT-HSCs was slightly increased in Carm1−/− FL, resulting in an increase in overall KLS cells (Fig. 5). This indicates that CARM1 is not required to maintain HSC cellularity in the FL. All other hematopoietic progenitors, from ST-HSCs through lymphoid committed CLPs and myeloid committed GMPs, as well as MEPs, were present in similar numbers to controls (Fig. 5). Although the lack of CARM1 did not reduce cellularity of LT-HSCs and subsequent hematopoietic progenitors (Fig. 5), we observed a striking decrease in hematopoiesis in the fetal BM, thymus, and spleen at the same time point. Therefore, we questioned whether the hematopoietic potential of Carm1−/− FL progenitors was impaired.

Carm1−/− FL cells have impaired hematopoietic potential in vivo

To assess the functional potential of E18.5 FL progenitors, we performed a competitive reconstitution experiment. We first crossed Carm1−/− mice with an actin-driven EGFP transgenic line (25). Similar to E18.5 FL, we did not observe a decrease in cellularity or in hematopoietic progenitor subsets in E14.5 FL (data not shown). Equal numbers of E14.5 FL cells from GFP+Carm1−/− embryos or GFP+ littermate controls were mixed with GFP− wild-type FL cells from E14.5 C57BL/6 embryos. This mixture was injected into sublethally irradiated Rag2−/−/γc−/− mice. A schematic of the experiment is shown in Fig. 6A. Recipient thymus and BM were analyzed for donor chimerism in all hematopoietic subsets 8–12 wk after transfer. Carm1−/− FL cells were dramatically impaired in their ability to give rise to all thymocyte subsets, from DN1 through CD4SP and CD8SP, when in competition with control FL cells (Fig. 6B). Furthermore, in this competitive setting, Carm1−/− FL cells failed to contribute efficiently in establishing hematopoietic progenitor chimerism in the BM, with the exception of MEP (Fig. 6C). Thus, despite the fact that hematopoietic progenitors were present at near-normal numbers in E18.5 Carm1−/− FL, they were severely impaired in their ability to contribute to hematopoiesis of all lineages. Given that the recipient mice were Carm1 sufficient, these data also demonstrate that CARM1 is required cell autonomously in hematopoietic progenitors.

Carm1−/− FL cells are capable of responding to Notch-driven T cell differentiation signals, but display reduced viability in vitro

The impaired ability of Carm1−/− FL cells to contribute to hematopoietic lineages, including T cells, suggests that they are compromised either in their differentiation potential or in their response to survival cues. Although there were insufficient numbers of Carm1−/− fetal BM progenitors to compare the frequency of apoptotic cells relative to controls, we observed a slight increase in apoptosis in ex vivo Carm1−/− FL KLS progenitors (data not shown). Therefore, to functionally assess the ability of Carm1−/− FL progenitors to respond to differentiation and survival cues, we used the OP9-DL1 coculture system, which robustly promotes T cell differentiation from hematopoietic progenitors, largely through activation of the Notch1 signaling pathway (30, 31). Equal numbers of KLS progenitors from control or Carm1−/− E14.5 FL were sorted into triplicate wells containing monolayers of OP9-DL1 stroma. After 6 d of culture in the presence of IL-7 and Flt3L, the cells were harvested and analyzed by flow cytometry to determine their ability to commit to the T cell lineage. Both control and
Carm1−/− progenitors were capable of T cell commitment, as evidenced by differentiation to the DN3 developmental stage. However, we found a significant reduction in the number of DN1, DN2, DN3, and DN4 cells recovered from wells plated with Carm1−/− progenitors compared with controls (Fig. 7A). Neither control nor Carm1−/− cells progressed to the DP stage in this timeframe. Although we consistently observed a block at the DN1 to DN2 transition in vivo (Fig. 1) (23, 24), this was not observed in the in vitro OP9-DL1 coculture system. Instead, there was a consistent decrease of ∼4-fold for all subsets derived from Carm1−/− relative to control progenitors. To clarify the basis for the reduction in cellularity, we assessed whether Carm1−/− progenitors were defective in survival or proliferation in culture. We analyzed the frequency of cycling cells in the OP9-DL1 cultures, using Vybrant Dye Cycle to assess DNA content. We did not find a significant difference in the percentage of cycling cells between Carm1−/− and control cells (Fig. 7B). However, there was a significant increase in the frequency of apoptotic cells in wells seeded with Carm1−/− progenitors compared with control wells (Fig. 7C). Because IL-7 is a critical survival factor during T cell differentiation, we considered the possibility that reduced IL-7R expression could account for the survival defect.
of Carml−/− progenitors. IL-7R expression was not reduced in E18.5 Carml−/− CLPs in the FL. However, IL-7R expression was diminished on the entire CD44+CD25+ thymocyte subset, including the c-Kit+ DN1 progenitors in Carml−/− compared with wild-type controls (Fig. 7D). After T lineage commitment and progression to the DN3 stage, there was little or no difference in IL-7R expression. Taken together, these data suggest that Carml is required for survival of hematopoietic progenitors, particularly at the earliest stages of T cell differentiation.

Discussion

Protein arginine methylation is a posttranslational modification involved in various cellular functions, including signal transduction, subcellular protein localization, transcriptional regulation, protein–protein interactions, and DNA repair (6). We previously reported that the absence of Carml results in impaired fetal thymopoiesis (24). In this study, we demonstrate that this defect is due not only to a requirement for Carml in T cell development, but also to a much earlier requirement for Carml in oligopotent fetal hematopoietic progenitors. Although the number of LT-HSCs and downstream KLS subsets was not reduced in Carml−/− E14.5 or E18.5 FL, competitive reconstitution assays revealed a deficit in the ability of Carml−/− FL cells to contribute to hematopoiesis. Carml is required cell autonomously in hematopoietic cells, as revealed by impaired differentiation of Carml−/− FL cells in a Carml-sufficient host, as well as by unimpeded differentiation of wild-type thymocyte progenitors in a Carml−/− thymic stromal microenvironment.

Our previous observation of reduced thymic cellularity in E18.5 Carml−/− mice (24), together with our current finding of fewer thymocyte progenitors in thymic rudiments as early as E12.5 (Fig. 3), suggested that there might be a block in prethymic hematopoiesis. During fetal development, hematopoiesis transitions from the liver to the BM, such that at E18.5, hematopoiesis could occur in both compartments (28, 32, and this study). Therefore, we analyzed the frequency and number of hematopoietic progenitors in both the E18.5 FL and BM. Interestingly, although a severe reduction in all c-Kit+ progenitors was observed in the BM, the FL did not recapitulate this phenotype. Indeed, there was a small, but significant, increase in LT-HSCs in the E18.5 Carml−/− FL. In addition, at E14.5, when the FL is the major site of hematopoiesis, lack of Carml did not result in altered FL progenitor numbers or frequencies (data not shown). However, the competitive FL reconstitution assays did reveal a functional defect in these E14.5 FL progenitors (Fig. 6). There are at least three possible explanations that could reconcile normal numbers of FL hematopoietic progenitors with their impaired function and the reduction in BM progenitor cellularity in Carml−/− embryos. First, Carml deficiency could impair the ability of hematopoietic progenitors to emigrate from the FL, thus resulting in an accumulation of these progenitors in the FL and a reduction in the BM. However, this possibility is unlikely because on FL transplantation, a functional defect in these progenitors is revealed despite their manual release from the FL. Although this does not rule out a possible emigration defect, this mechanism is not sufficient to account for functional defects in hematopoiesis. Second, the ability of FL hematopoietic progenitors to home to the BM could be impaired. Chemotactants and integrins are known to regulate cellular trafficking and localization of hematopoietic cells, including LT-HSCs. Carml could control expression of these molecules, impacting the ability of Carml−/− FL HSCs to migrate to the fetal BM (33). Finally, Carml deficiency could alter the ability of HSCs in the fetal BM to respond to molecular cues in the HSC BM niche, which regulate self-renewal, survival, and/or differentiation (34).

Reduced functionality of fetal hematopoietic progenitors could be sufficient to explain the reduction in thymic cellularity; however, our data suggest that Carml plays an additional role in regulating thymocyte differentiation and/or survival. At E18.5, DN1 thymocyte progenitors are not significantly reduced in Carml−/− embryos. However, DN2 and subsequent stages of thymocyte differentiation are severely impaired (Fig. 1). These data indicate that Carml is required for efficient transition between DN1 and DN2 stages in vivo, in keeping with our previous report (24). Interestingly, whereas Carml deficiency results in a marked deficit in E18.5 BM progenitors with the potential to seed the thymus (note the reduction in Flk2+CD27+ progenitors in Fig. 4) (29), there is not a significant reduction in DN1 numbers. Thus, DN1 niches may be extremely limiting, so that the reduced number of CLPs in the BM would still provide an adequate number of thymic seeding cells to saturate this niche. In addition, homeostatic mechanisms may be in place to maintain DN1 cellularity. Absence of such homeostatic factors in the OP9-DL1 culture system could account for the reduced DN1 cellularity in the Carml−/− versus control cultures (Fig. 7A). Furthermore, increased apoptosis of Carml−/− progenitors in vitro supports a role for Carml in maintaining survival during T cell differentiation (Fig. 7C). Because IL-7 is known to be a potent survival cue during T cell differentiation, the reduction in IL-7R expression on ex vivo Carml−/− DN1 thymocytes could account for the impaired survival and reduced cellularity in conditions promoting T cell differentiation (Fig. 7D). We note that Carml is not required for IL-7R expression in all hematopoietic progenitors because IL-7R expression is not impaired in FL CLP or lineage committed DN3 thymocytes. In contrast with its influence on survival, Carml is not essential for Notch1-driven T cell commitment, as evidenced by the ability of surviving DN1 progenitors to progress through subsequent DN2 and DN3 maturation stages (Fig. 7B). Taken together, our findings indicate that Carml influences hematopoiesis both in BM and FL progenitors, as well as in thymocyte progenitors, consistent with a role for Carml in differentiation or survival, or both, of multiple cell types (6).

As a member of the PRMT family of arginine methyltransferases, Carml contributes to epigenetic regulation of differentiation in many cell types (6). The enzymatic activity of Carml is required for thymocyte development, as well as for embryonic survival, adipocyte differentiation, and transcriptional coactivator activity (23). Thus, arginine methylation is a critical epigenetic modification that contributes to proper differentiation of hematopoietic lineages. Indeed, epigenetic regulation of HSC self-renewal and differentiation has been demonstrated by dynamic changes in the methylation status of both DNA and histones during hematopoietic lineage progression (35–37). In this study, we demonstrate a novel function for the epigenetic modifier Carml in the regulation of fetal hematopoiesis in BM and FL, as well as in thymopoiesis. Given this impact of Carml on early hematopoiesis and thymocyte development, Carml-mediated epigenetic regulation may contribute to lymphoid and myeloid leukemogenesis. In this light, small molecule inhibitors of Carml may have therapeutic potential. The feasibility of this approach is suggested by the finding that treatment of Th cells with broad-spectrum PRMT small molecule inhibitors partially blocks cytokine production (38). In conclusion, Carml is required at multiple stages of hematopoietic differentiation, identifying it as a key epigenetic regulator of a cellular differentiation process that occurs throughout life. Future investigations will further elucidate the role of Carml at specific stages of hematopoiesis and determine the potential for therapeutic modulation of Carml, which could be beneficial for hematopoietic disorders.
Acknowledgments

We thank Dr. Irving Weissman for providing the actin-EGFP mice. We also thank Pam Whitney for flow cytometric analysis and Hilary Selden for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References